Original articles

Changes in sputum composition between two inductions performed on consecutive days

O Holz, K Richter, R A Jörres, P Speckin, M Mücke, H Magnussen

Abstract

***Background* – Sputum induction is a non-invasive method for obtaining cellular and biochemical material from the airways and appears to be particularly suited for repeated testing. However, it has not been clarified whether repeated inductions lead to a change in sputum composition. The aim of this study was to compare induced sputum results between two inductions performed 24 hours apart.**

***Methods* – Ten subjects with mild asthma and 19 healthy subjects were included. Sputum was obtained during three consecutive 10 minute periods of hypertonic saline inhalation. Samples were analysed separately for the three inhalation periods. Corresponding pooled values were computed, taking into account total cell numbers of each inhalation period.**

***Results* – In the three consecutive inhalation periods mean (SE) percentages of neutrophils increased from 29.2 (4.2)%, 22.0 (4.6)% and 14.5 (2.9)% on day 1 to 43.1 (5.3)%, 34.8 (5.5)% and 25.7 (5.3)% on day 2 in healthy subjects and from 21.3 (4.3)%, 24.1 (5.9)% and 15.9 (3.7)% to 35.9 (6.9)%, 30.7 (7.1)% and 31.8 (6.5)% in asthmatic subjects. This parallel shift corresponded to a mean (95% CI) increase in the pooled percentages of neutrophils of 17.4 (11.6 to 23.3)% in healthy and 14.6 (1.2 to 28.0)% in asthmatic subjects. In contrast to neutrophils, the percentage of macrophages decreased from day 1 to day 2, while eosinophil and lymphocyte percentages did not change significantly.**

***Conclusion* – These results suggest that the induction procedure itself causes a change in the composition of sputum detectable after 24 hours. This effect has to be taken into account when repeated sputum induction is performed.**

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Keywords: neutrophils, eosinophil cationic protein, airway inflammation.

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Sputum induction by inhalation of hypertonic saline has gained increasing interest as a non-invasive tool for obtaining cellular and soluble factors from the airways. The method has been shown to be reproducible, sensitive and valid[1 2] and a series of technical factors has been elucidated which could influence the results.[3] It has not been ascertained, however, whether the induction procedure itself affects the composition of sputum samples obtained in a second induction.

This question is of interest because sputum analysis appears to be particularly suited to being performed repeatedly within a short time. However, data on the effects of repeated sputum inductions, without therapeutic interventions, are scarce and conflicting.[4–7] The aim of our study was therefore to compare sputum composition between two inductions performed 24 hours apart in healthy subjects and subjects with mild asthma. To ensure comparability with data in the literature we adhered to a previously validated induction procedure.[1 8]

Methods

SUBJECTS

Ten subjects with mild bronchial asthma (mean (SD) age 30 (9) years; forced expiratory volume in one second (FEV_1) 95 (8)% predicted[9]) and 19 healthy subjects (age 26 (5) years; FEV_1 105 (13)% predicted) were studied. The diagnosis of bronchial asthma followed internationally accepted criteria.[10] All subjects with asthma showed a positive skin prick test to at least one common allergen (Allergopharma, Reinbek, Germany) and were hyperresponsive to methacholine, their provocative concentrations ($PC_{20}FEV_1$) being less than 8 mg/ml (geometric mean (SD) 0.44 (3.9) mg/ml). None had taken inhaled corticosteroids within the preceding three months. Nine of the healthy subjects had a history of seasonal rhinitis and three were mildly hyperresponsive to methacholine ($PC_{20}FEV_1$ >3.0 mg/ml), whereas the remaining subjects were normoreactive. In all subjects with seasonal allergies tests were performed out of season. All subjects were non-smokers and had not suffered an upper respiratory tract infection within the four weeks preceding the study. The tests were approved by the ethics committee of the Chamber of Physicians of the State of Schleswig-Holstein and all subjects gave their written informed consent.

SPUTUM INDUCTION

Subjects visited the laboratory on three occasions. On the first visit (day 0) lung function

measurements and methacholine inhalation challenge[11] were performed. At least seven days later the subjects underwent sputum inductions (day 1) which were repeated at the same time on the subsequent day (day 2).

Before sputum induction subjects inhaled 200 μg salbutamol from a metered dose inhaler. Lung function was measured before and every five minutes during the procedure. Subjects inhaled hypertonic saline from an ultrasonic nebuliser (NE-U12, Omron, Tokyo, Japan; delivered amount 1.72 ml/min, mass median diameter 4.9 μm) during three consecutive 10 minute periods. Sputum samples produced after the first (3% saline), during and after the second (4% saline), and during and after the third 10 minute inhalation period (5% saline) were processed separately. Subjects were asked to rinse their mouth thoroughly, blow their nose, swallow water, and then to expectorate the sputum onto a plastic petri dish. Samples were kept at 4°C and processed during the induction procedure or immediately afterwards.

SPUTUM PROCESSING

All adequate plugs of sputum were separated from saliva,[8] plugs being defined as the dense and viscid parts and considered to be adequate if containing non-squamous cells and little or no squamous cell contamination.

Plugs were weighed and suspended in twice their volume of sputolysin (6.5 mM dithiothreitol, Calbiochem, Bad Soden, Germany). After incubation at 37°C for 15 minutes with repeated mixing, phosphate buffered saline (PBS) was added to achieve a 20-fold final dilution. Samples were centrifuged at 600 *g* and supernatants were collected and stored at −80°C until required for further analysis. Cells were resuspended in PBS/BSA (1%), cell counts were measured in a haemocytometer, viability was determined by trypan blue exclusion, and a portion of 30 000–60 000 cells was used for cytocentrifuge preparations.

Differential cell counts were assessed by two observers from 400–500 non-squamous cells on coded Giemsa stained slides. Their mean results were expressed as a percentage of the total non-squamous cell numbers. The concentration of eosinophil cationic protein (ECP) was analysed by the Pharmacia CAP system (Pharmacia and Upjohn, Erlangen, Germany) in the 20-fold diluted samples and the concentration in the original sample was recalculated.

To achieve comparability with conventional sputum analysis, in which consecutive samples are pooled,[12] we also computed equivalent pooled values of total cell numbers and concentrations of ECP per ml of sputum by taking into account the weight of the respective sputum plugs. Pooled percentages of cells were derived using the absolute cell numbers of samples from each inhalation period.

STATISTICAL ANALYSIS

Arithmetic mean (SE) values of percentage cell numbers were computed, as well as mean values and 95% confidence intervals of differences in pooled values. Using log and antilog transformation, geometric mean values were computed for numbers of cells per ml and ECP concentrations to take account of the skewed data distribution. Geometric SE was computed as a factor of geometric mean value after antilog transformation of the SE computed from log transformed values. Correspondingly, mean changes and 95% confidence intervals of log transformed pooled values were expressed as percent changes on day 2 relative to day 1. Analysis of variance (ANOVA) was performed using the three consecutive samples within each sputum induction, with day and inhalation period as within subjects factors. This type of statistical comparison between days comprised a simultaneous comparison of the three inhalation periods. Data of cell numbers per ml sputum and ECP concentrations were logarithmically transformed to fulfil the requirements imposed by ANOVA. The level of statistical significance was set at $p<0.05$.

Results

In the consecutive sputum inhalation periods five of the 60 single samples could not be

Table 1 Data on sputum composition for the three consecutive inhalation periods

	Inhalation period	Healthy subjects		Asthmatic subjects	
		Day 1	Day 2	Day 1	Day 2
Viability (%)§	1	84 (3)	88 (4)	81 (4)	79 (7)
	2	86 (3)	88 (3)	87 (3)	91 (2)
	3	80 (5)	85 (4)	84 (4)	85 (2)
Squamous cell contamination (%)§	1	3.4 (1.4)	7.1 (2.2)	3.9 (1.2)	5.8 (1.5)
	2	3.2 (0.7)	4.1 (1.5)	3.1 (0.7)	2.7 (1.0)
	3	3.9 (0.8)	5.5 (1.5)	4.3 (1.3)	6.5 (1.6)
Total cell count of non-squamous cells (10^6/ml)#	1	8.5 (1.2)	6.9 (1.4)	4.0 (1.6)	7.4 (1.4)
	2	6.5 (1.3)	7.9 (1.2)	3.3 (1.4)	3.5 (1.7)
	3	3.5 (1.3)	3.7 (1.3)	2.4 (1.4)	2.0 (1.4)
Neutrophils (10^6/ml)#	1	1.86 (1.4)	3.40 (1.5)	0.70 (1.7)	2.15 (1.6)
	2	0.97 (1.5)	2.20 (1.4) *	0.60 (1.4)	1.20 (1.8) *
	3	0.34 (1.4)	0.65 (1.5)	0.40 (1.4)	0.70 (1.5)
ECP (μg/l)#	1	389 (1.4)	513 (1.7)	485 (1.4)	713 (1.5)
	2	186 (1.4)	323 (1.5) *	292 (1.5)	383 (1.5) *
	3	98 (1.3)	122 (1.4)	127 (1.3)	283 (1.3)

§ Values are given as mean and standard error of mean.
# Values are given as geometric mean and geometric standard error (factor, see methods).
* $p<0.05$ day 1 versus day 2 (ANOVA).

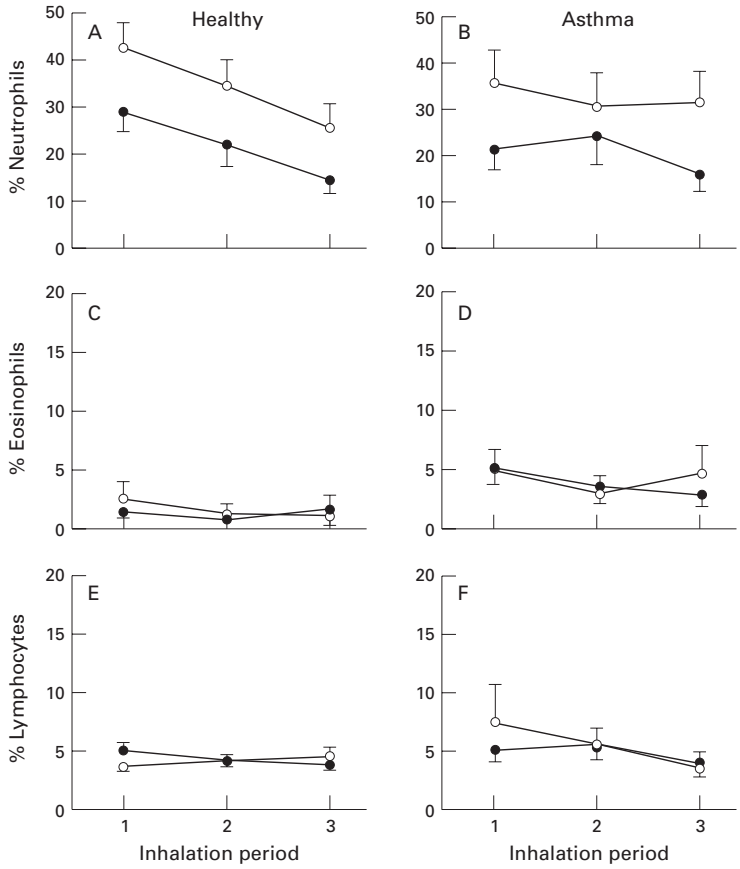

Figure 1 Mean (SE) percentage of neutrophils (A, B), eosinophils (C, D), and lymphocytes (E, F) in induced sputum measured on day 1 (●) and 24 hours later on day 2 (○). Inhalation periods 1–3 indicate consecutive 10 minute sampling periods within each of the two sputum inductions. Note the different scales. The increase in the level of neutrophils in the second compared with the first sputum induction was statistically significant in both healthy and asthmatic subjects (p<0.01 each).

evaluated in the asthmatic subjects and seven of the 114 samples in the healthy subjects. The mean (SD) maximum fall in FEV_1 during sputum inductions was 2.7 (1.9)% in the healthy subjects and 4.7 (4.1)% in the asthmatic subjects and did not differ between days 1 and 2.

In both groups squamous cell contamination and viability were not significantly different between days 1 and 2 (table 1). Pooled total numbers of all non-squamous cells per ml sputum increased by a mean (95% CI) of 17 (−17 to 66)% in healthy subjects and 16 (−35 to 107)% in asthmatic subjects between days 1 and 2.

The separate analysis of the three consecutive inhalation periods revealed that percentages of neutrophils were increased on day 2 compared with day 1 in healthy and asthmatic subjects (ANOVA, p<0.01 each; fig 1A and B). Correspondingly, percentages of macrophages decreased (ANOVA, p<0.01 each). Percentages of eosinophils and lymphocytes did not change significantly between days 1 and 2 (fig 1C–F). To enable the comparison with published data, pooled percentages of cells (table 2) and differences between days 1 and 2 were computed from the consecutive samples. Mean (95% CI) differences in percentage of neutrophils between days 1 and 2 were 17.4 (11.6 to 23.3)% in healthy subjects and 14.6 (1.2 to 28.0)% in asthmatic subjects. Corresponding values for pooled percentage macrophages were −17.7 (−23.0 to −12.4)% in healthy subjects and −11.9 (−26.6 to 2.9)% in asthmatic subjects. Pooled percentage eosinophils and lymphocytes did not differ significantly between days 1 and 2.

The separate analysis of the three consecutive inhalation periods revealed that the total numbers of neutrophils per ml sputum were significantly raised on day 2 compared with day 1 in both groups (ANOVA, p<0.05 each; table 1). Pooled total numbers of neutrophils per ml sputum were increased on average (95% CI) by 94 (37 to 179)% in healthy subjects and by 114 (7 to 329)% in asthmatic subjects. The total numbers of macrophages, eosinophils, and lymphocytes did not differ significantly between the two days.

Separate analysis of consecutive sputum samples showed that the concentrations of ECP were significantly raised on day 2 compared with day 1 in both healthy and asthmatic subjects (ANOVA, p<0.05 each; table 1). Pooled concentrations of ECP increased by a mean (95% CI) of 58 (12 to 122)% in healthy subjects and 57 (−13 to 181)% in asthmatic subjects.

Table 2 Mean (SE) data on pooled sputum composition

	Healthy subjects		*Asthmatic subjects*	
	Day 1	*Day 2*	*Day 1*	*Day 2*
Macrophages (%)	68.90 (3.11)	51.20 (4.68) **	63.71 (4.79)	51.84 (3.84)
Neutrophils (%)	24.34 (3.24)	41.77 (5.00) **	20.93 (5.03)	35.55 (4.34) *
Eosinophils (%)	0.99 (0.39)	1.46 (0.76)	3.66 (0.89)	5.21 (1.12)
Lymphocytes (%)	4.42 (0.50)	3.92 (0.42)	4.61 (0.93)	5.88 (1.62)

** p<0.01, * p<0.05 day 1 versus day 2.

Discussion

Our results demonstrate a marked increase in neutrophil numbers in induced sputum 24 hours after a previous sputum induction, both in healthy and mild asthmatic subjects. In contrast, the numbers of other cell types including eosinophils were not altered. Fluid phase concentrations of ECP, however, were increased in the second sputum induction. These data suggest that the procedure of sputum induction itself can cause an alteration in the composition of induced sputum.

Few studies have been performed of repeated sputum testing without intermediate therapeutic interventions. Fahy *et al* reported that no changes in sputum composition occurred 20 hours after a previous induction, although the range of the percentage of neutrophils in eight asthmatic subjects was increased.[7] Furthermore, sputum inductions have been performed on five consecutive days without changes in sputum composition.[4] In contrast, Kips *et al* found an increase in the percentage of neutrophils on two consecutive days[5]; this study, however, included a methacholine challenge between the sputum inductions. Other investigators have reported a decrease in the percentage of macrophages within four repeated sputum inductions in a small sample of

healthy subjects.[6] Our study compared two inductions on two consecutive days in larger groups of subjects and did not incorporate any other intervention which might have affected the results.

The increase in percentage and absolute numbers of neutrophils in the second sputum induction, as shown for the separate as well as the pooled analysis of samples, and the fact that absolute numbers of macrophages and other cell types were unchanged, suggested an influx of neutrophils after the first sputum induction. The mean change in percentage neutrophils by 17.4% and 14.6% in healthy and asthmatic subjects is compatible with the mean increase in the total numbers of all cells by 17% and 16%, respectively, although the latter was not statistically significant, probably because of the higher variability of this parameter. It is possible that the inhalation of hypertonic saline was responsible for the neutrophil influx, either because of the amount of fluid deposited in the airways, as suggested by the increase in neutrophils observed 2–7 hours after a bronchoalveolar lavage using isotonic saline,[12 13] or because of a change in the osmolarity of the epithelial lining fluid. The time course of the neutrophilic response within the airways is not clear. Segmental challenges with isotonic or hypertonic saline indicated that neutrophil numbers are not altered five minutes after the challenge.[14] It is therefore not likely that cell numbers are already affected during a single sputum induction. This is in line with the finding that the composition of spontaneous sputum does not differ from that of induced sputum.[15] However, it was not the aim of our study to clarify the mechanisms underlying the neutrophilic response.

Baseline levels of ECP in induced sputum were only slightly raised in the subjects with mild asthma compared with healthy controls. The fact that ECP levels were increased on day 2 in both groups suggests a similar release of ECP induced by the first sputum induction.

We followed a widely used protocol for sputum induction which employs inhalation of increasing concentrations of hypertonic saline (3–5%) for 7–10 minutes each[1 8] and selection of sputum plugs. It has not been investigated whether other procedures such as inhalation of 3% saline for 20 minutes[16] or the use of nebulisers with a lower output produce the same effect as that observed in our study. In single inductions similar results are obtained between different induction methods in terms of cellular composition or lung function responses to saline inhalation.[3] In a departure from the common procedure, however, we decided to analyse the samples of the three consecutive inhalation periods within each sputum induction separately in order not to miss any changes which might have been obscured with pooling of samples. In a previous study we found that there was a significant fall in the percentage of neutrophils during sputum induction,[17] possibly resulting from the fact that the material originated from different levels within the airways. As an additional benefit of the separate analysis of the consecutive sputum samples, the statistical power of the study was increased. The fact that the changes induced by the first sputum test showed a parallel shift in the separate analysis of consecutive samples (fig 1A and B) was compatible with our finding that changes of the same direction and magnitude were observed in pooled samples which had been derived from the separate samples by computing weighted average values.

In summary, our data indicate that samples of induced sputum obtained 24 hours after a previous induction differ in neutrophil count and ECP concentration from samples obtained in the first induction, thereby suggesting that care should be taken when applying repeated sputum testing in healthy or mild asthmatic subjects.

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