Induced sputum cell and fluid-phase indices of inflammation: comparison of treatment with dithiothreitol vs phosphate-buffered saline

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ABSTRACT: Treatment of sputum with dithiothreitol (DTT) gives reliable measurements of cellular and fluid-phase markers of airway inflammation. We investigated the extent to which DTT treatment influences these measurements as compared with phosphate-buffered saline (PBS).

Hypertonic saline-induced sputum, collected from 20 asthmatic subjects, was examined within 2 h. All portions which looked more solid (less fluid) than saliva were collected from the expectorate. The selected sputum was then divided into two portions: one treated with one volume of DTT and one volume of PBS, the other with two volumes of PBS. The filtrates were assessed blind for total and differential cell count, viability, and fluid-phase eosinophil cationic protein (ECP), fibrinogen, interleukin (IL)-5 and IL-8.

Sputum treated with DTT compared with PBS had lower proportions of viable cells (median 66 versus 74%; $p=0.003$). In contrast, DTT-treated sputum had higher total cell counts (median 8.8 vs 2.8×10^6 mL⁻¹; $p<0.001$) and levels of ECP (median 1340 vs 584 mg·L⁻¹; $p<0.001$). The measurements were similar with respect to the proportion of eosinophils, neutrophils, lymphocytes, macrophages, and fluid-phase fibrinogen, IL-5 and IL-8.

We conclude that dithiothreitol disperses cells more effectively and that this might account for the higher levels of eosinophil cationic protein. Dithiothreitol may affect cell viability, but the changes are not relevant with respect to cell counts. Additionally, dithiothreitol does not seem to influence the other measurements performed.

Eur Respir J 1997; 10: 1336–1340.

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Keywords: Airway inflammation
asthma
dithiothreitol
induced sputum

Received: June 24 1996
Accepted after revision February 12 1997

Supported by grants from Astra Pharma Inc. and Boehringer Ingelheim (Canada) Ltd.

Sputum examination has become an important method of assessing airway inflammation directly and relatively noninvasively. Two major developments have motivated the recent interest in this method: the induction of sputum with an aerosol of hypertonic saline in asthmatics unable to produce it spontaneously [1]; and improvements in the method of sputum examination to produce reliable results [2–4]. The latter have included: the use of dithiothreitol (DTT) to disperse cells [5]; the examination of cells on cytopins [6]; the collection of supernatant for fluid-phase measurements [4, 6, 7]; and the evaluation of the necessary properties required by a method to be applied in research or clinical practice, such as repeatability, validity [4] and responsiveness [8–10].

In 1964, CLELAND [11] identified DTT and its isomer dithioethreitol as agents with low redox potential, which could reduce and split glycoprotein disulphide bonds. Subsequently, SHAH and DYE [12] showed that this property allowed dispersion of sputum before the processing of smears and cultures to identify mycobacteria. However, these agents were not used to process sputum for inflammatory cell examination until 1978 [5]. Sputum treatment with DTT did not affect cell morphology [5], and exposure to DTT for up to 30 min did not affect

differential cell counts [3]. However, these studies were uncontrolled [3], or used a negative control, and the effects of DTT on the fluid-phase markers were not examined.

We have, therefore, investigated the effect of sputum selected from the remainder of the expectorate [13] treated with DTT, as compared with phosphate-buffered saline (PBS), on the cell content and fluid-phase levels of eosinophil cationic protein (ECP), fibrinogen, interleukin (IL)-5 and IL-8. Each sample of sputum was divided into two portions: one treated with four volumes of DTT followed by four volumes of PBS, and the other with eight volumes of PBS alone. The measurements in the filtrates were performed blind to sputum or subject characteristics.

Methods

Subjects

The study population comprised 20 asthmatic patients recruited from the Firestone Regional Chest and Allergy Clinic (table 1). Asthma was identified by episodic wheezing, chest tightness and dyspnoea, and either by:

Table 1. – Characteristics of subjects

Subjects n	20
Gender male/female	12/8
Age yrs	49 (12–69)
Smoking current/ex	1/11
Atopic*	12
FEV ₁ L	2.5 (1.1–4.1)
% pred	78.2 (50.0–130.0)
PC ₂₀ mg·mL ⁻¹ †	1.4 (0.07–16)
Steroids inhaled/oral‡	15/4

Data are presented as absolute number or as mean, and range in parenthesis. *: atopic means one or more positive allergy skin-prick test; †: geometric mean; ‡: inhaled steroid was beclomethasone dipropionate or budesonide and oral was prednisolone. FEV₁: forced expiratory volume in one second; % pred: percentage of predicted value; PC₂₀: provocative concentration of methacholine causing a 20% fall in FEV₁.

reversible airflow limitation (forced expiratory volume in one second (FEV₁) <70% predicted or previous best, which increased by ≥15% after 200 µg of salbutamol) (n=8); or by methacholine airway hyperresponsiveness (provocative concentration producing a 20% fall in FEV₁ (PC₂₀) <8 mg·mL⁻¹) if the FEV₁ was >70% pred (n=12). The study was approved by the Hospital Research Committee and all subjects gave written informed consent.

Study design

This was a cross-sectional study, where samples of induced sputum were divided into two portions and treated with either DTT followed by PBS or with PBS alone. The samples were processed simultaneously by two observers, with a high interobserver repeatability on sputum handling and counting [14]. Cells and fluid-phase measurements were performed blind on coded filtrates.

Clinical methods

Subjects were screened by means of a questionnaire, spirometry and a methacholine inhalation test (if the FEV₁ was >70%). Spirometry was performed with a PK 131 Morgan Spiroflow dry rolling-seal spirometer (Roxon Medi-Tech, Rexdale, Ontario, Canada). Baseline measurements of slow vital capacity (VC) and FEV₁ were made according to American Thoracic Society (ATS) criteria [15], and reference values were taken from CRAPO *et al.* [16]. Methacholine inhalation tests and allergy skin tests, with 19 common allergen extracts, were performed using standard procedures [17, 18].

Sputum induction was performed with an aerosol of hypertonic saline using a modification of the method of PIN *et al.* [1]. The aerosol was generated by a Fisoneb Ultrasonic nebulizer (Canadian Medical Products Ltd, Markham, Ontario, Canada) with an output of 0.87 mL·min⁻¹ and particle size of 5.58 µm aerodynamic mass median diameter. Increasing concentrations of saline (3, 4 and 5%) were inhaled *via* a mouthpiece, without a valve or noseclips, for 7 min. After each inhalation, a FEV₁ value was measured for safety, the mouth was rinsed with water, water was swallowed, and the nose was blown to reduce contamination of the specimen with saliva and postnasal drip. An attempt was then made to cough sputum into a sterile container.

Sputum samples were examined as soon as possible within 2 h, using the method described by PIZZICHINI *et al.* [4]. The expectorated specimen consisting of sputum and saliva was poured into a Petri dish, and all portions which looked more solid (less fluid) than saliva were carefully selected and divided into two portions of similar weights. They were clear or opaque and had little or no squamous epithelial cell contamination. Both portions were examined simultaneously by two observers. One portion was weighed and treated with a volume of DTT (diluted to 0.1% by the addition of distilled water (Sputolysin 10%; Calbiochem Corp., San Diego, CA, USA)) equal to four times this weight (*i.e.* if the weight was 1 mg, 4 mL of DTT was added). The treated sputum was rocked for 15 min to break up the mucus. A further four volumes of PBS was then added, and the rocking was continued for 5 min to slow down the action of the DTT. The final concentration of DTT in the fluid recovered was 0.05%. The other portion was weighed and treated with eight volumes of PBS for 20 min. Each mixture was filtered through a 48 µm nylon mesh (B&SH Thompson, Mississauga, Ontario, Canada). A total cell count was obtained with a modified Neubauer counting chamber and cell viability was determined by trypan blue exclusion. Cytospins were prepared, treated with Wright's stain and a 400 nonsquamous cell differential cell count was performed. The remaining cell suspension was cytocentrifuged at 790×g for 4 min and the supernatant was collected and stored at -70°C for later assay of ECP, fibrinogen, IL-5 and IL-8. The concentrations of ECP (µg·L⁻¹), IL-5 (pg·mL⁻¹), and IL-8 (pg·mL⁻¹) in the thawed supernatant were determined using a sensitive radioimmunoassay (RIA; Kabi Pharmacia Diagnostics AB, Uppsala, Sweden) and fibrinogen (ng·mL⁻¹) was measured by a "sandwich" enzyme-linked immunosorbent assay (ELISA) using a rabbit anti-human fibrinogen antibody (Dako No. A080) [4].

Statistical analysis

Results are summarized as median and interquartile range. Data with non-normal distribution (total cell counts, eosinophils, lymphocytes, ECP, fibrinogen, IL-5 and IL-8) were log-transformed before analysis. Differences between samples were assessed by paired t-test. Correlations were assessed by the Spearman rank correlation coefficient. The effect of DTT on the slopes of the curves for the correlations was compared using regression analysis, with the DTT treatment as a covariate. Significance was accepted at the level of 95%.

Results

The DTT-treated portions were compared with the portions treated with PBS alone (figs. 1 and 2 and table 2). They had a higher total cell count and ECP levels, and lower cell viability. The absolute eosinophil counts correlated positively with levels of ECP in both portions, and the correlations were not influenced by the DTT treatment. The differential cell count of eosinophils, neutrophils, lymphocytes and macrophages and the levels of fluid-phase fibrinogen, IL-5 and IL-8 were not different (table 2).

Fig. 1. – The difference (in logs, except for cell viability) between sputum treated with DTT and PBS on: a) cell viability; b) total cell count; c) absolute count of eosinophils; and d) ECP, plotted against the mean of the two values. DTT: dithiothreitol; PBS: phosphate-buffered saline; ECP: eosinophil cationic protein.

Fig. 2. – Correlations between absolute count of eosinophils in sputum and fluid-phase ECP in: a) DTT-treated and b) PBS-treated samples. r_s : Spearman rank correlation coefficient. The solid line is the calculated linear regression. Highly significant correlations were observed in both portions of sputum between the absolute counts of eosinophils and ECP. For definitions see legend to figure 1.

Table 2. – Weight and values of inflammatory indices in sputum treated with DTT and PBS

	DTT		PBS		p-value
Weight mg	354	(183)	412	(135)	NS
Total cell count $\times 10^6$ cells.mL ⁻¹	8.8	(20)	2.8	(6.7)	0.001
Viability %	66	(21)	74	(19)	0.003
Eosinophils %	6.2	(20)	2.7	(12)	NS
Neutrophils %	52.9	(34)	60.7	(52)	NS
Lymphocytes %	1.0	(1)	1.4	(1)	NS
Macrophages %	28.4	(29)	19.3	(24)	NS
ECP μ g.L ⁻¹	1340	(2665)	584	(3392)	0.001
Fibrinogen μ g.L ⁻¹	5920	(11720)	3760	(6220)	NS
IL-5 ng.L ⁻¹	464	(444)	368	(568)	NS
IL-8 μ g.L ⁻¹	13600	(95440)	13600	(20740)	NS

Data are presented as median and interquartile range in parenthesis. DTT: dithiothreitol; PBS: phosphate-buffered saline; ECP: Eosinophil cationic protein; IL: interleukin; NS: non-significant.

Discussion

In this study, portions of sputum were selected from the remainder of the expectorate. We have previously shown that this selected portion is minimally contaminated with saliva, whilst the remainder of the expectorate is mainly saliva contaminated with some sputum [13]. The selected sputum, when treated with DTT, resulted in a threefold higher cell recovery and a twofold higher level of fluid phase ECP than selected sputum treated with PBS alone. Viable cells were obtained in

both portions, but cell viability was lower in the DTT-treated samples. The differential cell count and fluid-phase concentrations of fibrinogen, IL-5 and IL-8 were similar. The results of this study confirm that DTT breaks up mucus and disperses the cells much more effectively than PBS, and suggest that treatment of sputum with DTT does not adversely affect the cell counts or the fluid-phase indices measured.

This is the first comparison of inflammatory cells and fluid-phase markers between induced sputum treated with DTT or PBS. The comparison is important, since current methods of sputum examination involve the use of DTT to disperse cells, and its effects on soluble markers have not been investigated. Our results on inflammatory cells confirm and extend the initial observations of WOOTON and DULFANO [5]. They are also in keeping with a previous examination of the effects of the exposure time to DTT on sputum total and differential cell counts [3]. The exposure of sputum to 0.1% DTT for increasing periods of 10, 20 and 30 min increased the yield of cells and improved the quality of cytopsin preparations. The reason for the lower viability of cells in the DTT-treated samples is unknown. Because it does not affect cell morphology [19] or differential cell counts [20], it may be falsely elevated in the PBS-treated samples due to the low recovery of cells. Whatever the reason, this is not important for cell counts because the cell viability, although lower, was still good in the DTT-treated sputum.

We found no effect of DTT on fluid-phase fibrinogen, IL-5 and IL-8. This is useful information, since DTT, by breaking disulphide bonds [11], could result in falsely low readings of cytokines or other inflammatory proteins, such as ECP and fibrinogen. Our results indicate that these effects are not relevant, at least for the indices measured in the way DTT was used and neutralized.

The fluid-phase levels of ECP were higher in DTT-treated samples. This could be due to a higher recovery of ECP, which is separated into the airway mucus, or alternatively to the activation of eosinophils or release of ECP from eosinophils by DTT. The first possibility is supported by the results of this study, showing an equally good correlation, without evidence of outliers, between the absolute number of eosinophils and the ECP levels in the supernatant in sputum treated both with DTT or PBS. It is also supported by a previous study [13], in which sputum selected from the expectorate was compared to the remainder (composed mostly of saliva). Whilst there was a loss of less than one third of the sputum (nonsquamous) cells from the selected portion compared to the remainder, the selected portion had a sixfold higher fluid phase concentration of ECP, suggesting that most of the ECP is contained within the mucus and that DTT is needed to release it. Further investigation is required to investigate the possibility that DTT may activate eosinophils or otherwise release ECP from eosinophils.

Two methods to examine sputum for inflammatory indices have been used. In one, sputum is selected from saliva [13], whilst in the other the whole expectorate of sputum plus saliva is examined [7]. The results of this study are valid for the selection method which has been described and evaluated in more detail. In this method,

the influence of other confounders has been investigated [3, 14, 21, 22], and the measurements have been shown to be highly reproducible [4]. This implies that the results of this study are actually due to the effect of sputum with DTT or PBS, and not to other confounders in the method.

In conclusion, treatment of all portions of induced sputum (selected from the remainder of the expectorate) with dithiothreitol 0.1% for 15 min followed by phosphate-buffered saline does not adversely affect cell counts or fluid-phase levels of eosinophil cationic protein, fibrinogen, interleukin-5 and interleukin-8 of asthmatic subjects. However, it does lead to a higher recovery of cells and eosinophil cationic protein.

Acknowledgements: The authors wish to thank the subjects who volunteered to help with this study, S. Weston for helping with cell counts, S. Evans for performing the fluid-phase measurements and Pharmacia Diagnostics AB, Uppsala, Sweden for providing the ECP kits.

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