

EDITORIAL

Optimizing sputum methodology

The analysis of sputum has gained increasing acceptance as a means of assessing airway inflammation since its first description [1, 2]. Sputum induction by the inhalation of nebulized hypertonic saline has been demonstrated to be a relatively safe and effective method of enabling sputum expectoration [3, 4]. Sputum processing generally consists of selecting the solid mucinous portion of sputum, that is liquefied by reduction with dithiothreitol, and using the cellular portion to generate cytopins for light microscopy, while employing the supernatant for analysis of soluble proteins and mediators. In response to the expanding volume of sputum research, the European Respiratory Society has set up an international task force to standardize sputum induction and processing methodology, and their report is now near completion. As a refinement to this methodology, Dr Maria C. Ronchi and colleagues describe modifications to sputum processing in this issue of *Clinical and Experimental Allergy*. They propose stepwise sequential filtration with nylon net filters, Percoll density gradient centrifugation, and the use of cell culture medium; in this way cytopins of improved quality are obtained with a cell suspension amenable to specialized analyses [5].

Historical perspective

In Ancient Greece phlegm was regarded by Hippocrates as one of the four humours fundamental to disease. Yet it was not until the nineteenth century that microscopy was used to demonstrate the presence of sputum eosinophilia in asthma [6], that was later shown to be associated with Charcot-Leyden crystals and Curschmann's spirals [7]. In the 1950s sputum microscopy was used in the diagnosis and monitoring of asthmatic patients by researchers such as French Hansel [8] and Morrow Brown [9]. More recently, F. E. Hargreaves and his team in Hamilton, Ontario have done most to establish sputum analysis in clinical practice; including providing detailed methods of sputum induction, processing and microscopy [10]. Nevertheless, a series of reviews and editorials reflect the considerable variations in methodology still present [4, 11–19].

Safety of sputum induction

In the clinical context of the patient, pretreatment with a bronchodilator, the duration of sputum induction, the output of the nebuliser, and the tonicity of saline all influence the frequency of bronchospasm during sputum induction [4, 20, 21]. The mechanism of this effect may involve airway smooth muscle contraction provoked by the release of tachykinins from sensory neurons or release of mediators from mast cells [22, 23]. However, despite bronchoprotection, it has been demonstrated

in a retrospective review of 351 inductions that 12% of asthmatic subjects developed bronchospasm [20]. In a study in severe asthma, sputum induction had to be stopped due to side-effects in 12% of patients [24]. Furthermore, sputum induction has been performed in asthmatic patients with an FEV₁ of <60% of predicted [3], while isotonic saline has been used in adult acute severe exacerbations of asthma [25]. In addition, the safety of sputum induction has been investigated in a multi-centre study [26], showing an FEV₁ decrease of $\geq 20\%$ from the postbronchodilator baseline in 14% of all subjects and in 25% of subjects whose initial prebronchodilator baseline was 40–60% of predicted. In these studies subjects with bronchoconstriction induced by saline generally responded promptly to β_2 -agonist treatment.

Sputum induction methodology

Induced sputum from asthmatics yields better quality cytopins with less squamous cell contamination than spontaneous sputum; although the leucocyte differential is similar [27]. The hypertonicity of inhaled saline does not affect sputum cell composition [28], but may cause reduced IL-8 levels [29]. Analysis of sequential induced sputum samples has shown that the percentages of eosinophils and neutrophils are significantly higher at the beginning of the sputum induction than at the end [30–32], these differences in sputum composition being more pronounced in healthy subjects than in asthmatic or COPD patients [33]. Studies have compared two sequentially induced sputum samples performed 24 h apart in healthy and asthmatic subjects [34, 35], and shown a significant increase in the proportion of neutrophils with a decrease in macrophages at 24 h. Sputum induction is not possible in all subjects, especially in children, and is not established in routine clinical practice.

Cell viability

Sputum should be processed as soon as possible, to minimize degeneration and maximize cell viability. However, sputum specimens can be kept at 4 °C if processed within 2 h. In selected induced sputum from asthmatic subjects that has been liquefied by dithiothreitol (DTT), cell viability ranged from 65 to 80% [27, 36]. In general, lower cell viability is found in spontaneous sputum both in asthma and COPD [27].

Sputum selection

The solid mucinous portion of sputum is generally selected from saliva [1, 2, 37, 38], although whole expectorate is analysed by

some workers [39,40]. The physical properties of some samples make it difficult to separate sputum from saliva even with the use of a stereoscopic or inverted microscope. Salivary contamination, residual mucus, cell debris and large squamous epithelial cells (40–60 µm diameter) can prevent reliable cell counting [41, 42]. Different upper limits of squamous cell contamination have been set to define an adequately selected sputum sample, ranging from 20 to 50% [43, 44]. Selected sputum enables better quality cytopspins to be produced, with less squamous cell contamination [16, 41, 45]. A higher percentage of eosinophils and higher levels of eosinophil cationic protein (ECP) were found in selected sputum as compared with the entire sample in asthmatics [46]. Selected samples had better slide quality and no difference in the differential cell count of eosinophils, neutrophils, and lymphocytes, with higher levels of ECP [45]. However, both the selected sputum and the entire sputum method have the same diagnostic value in distinguishing asthmatic patients from healthy subjects.

Dithiothreitol liquefaction

The examination of unprocessed smears has the disadvantage of sampling only a circumscribed part of the sputum. In addition, leucocytes may be entrapped in mucus and aggregated, making them difficult to identify. The introduction of DTT to release the entrapped cells, and the use of cytopspins to generate a monolayer of sputum cells [47] has made the quantification and characterization of cells more reliable [37]. DTT reduces the disulphide bonds which cross-link mucinous glycoproteins and maintain sputum in its gel form [48, 49], and causes minimal cell damage [50, 51]. Following liquefaction, cells are collected by centrifugation and the supernatant containing DTT is stored for soluble mediator assays.

DTT, while useful for obtaining cells, may interfere with immunological detection techniques. Many of the soluble mediators of interest measured in induced sputum contain disulphide bonds within their structure. This makes them susceptible to denaturation by DTT thus potentially altering epitopes and the immunological detection of these proteins. In addition, DTT may affect the antibody components of the immunoassay. Some workers have investigated the effects of DTT on immunological assays with sometimes contradictory results. Several reports have shown effects of DTT on the immunological detection of cell surface markers by flow cytometry [52–54].

ECP, EPO, MPO

ECP, eosinophil protein X (EPX), eosinophil peroxidase (EPO) and myeloperoxidase (MPO), are granulocyte granule proteins commonly measured in sputum. The detectable levels of EPO and MPO, but not ECP and EPX, were significantly reduced in sputum samples treated with DTT, compared with homogenization by ultrasound [55]. In contrast, increased levels of ECP have been noted after DTT treatment compared to phosphate-buffered saline [56, 57]. The sensitivity of EPO and MPO to DTT may be due to the presence of disulphide bonds in the tertiary structure [58].

IL-8

Chemokines and cytokines have been assessed in sputum in a number of clinical studies, and IL-8 has been measured in a range of lung diseases [57, 59–63]. Some studies have found no significant difference between standard samples of IL-8 assayed with and without DTT [36, 57, 60], yet other studies report IL-8 ELISAs as being linearly affected by DTT [64–66].

IL-5

An elegant methodological study of IL-5 measurement in sputum supernatants identified considerable technical problems due to DTT [67]. A range of validation experiments demonstrated that DTT did not interfere with the ELISA and that the IL-5 molecule remained intact, but that the recovery of immunoreactive spiked IL-5 was poor. Recently, a subsequent study by the same group suggests that proteolytic activity in the sputum sample may be interfering with the IL-5 measurements. Addition of protease inhibitors significantly increased the levels of detectable IL-5 [68].

Reproducibility and repeatability

Differential cell counts on sputum cytopspins have good inter-observer consistency [37, 38, 69, 70]. Similarly, the repeatability of differential cell counts and the measurement of soluble mediators in samples, obtained on different days from clinically stable patients has been reported to be good [1, 37, 69, 71–73]. In a multicentre study the reproducibility of measurements of cellular and fluid phase parameters in asthmatics were similar and without any significant centre effects [37]. The cell fraction and the fluid phase of induced sputum differ between asthmatics, smokers with COPD, and healthy subjects, indicating that sputum analysis can be used to assist the diagnosis of these conditions [2, 37, 74].

Induced sputum, bronchoalveolar lavage and bronchial biopsies

The relationship between the cellular content in sputum and airway tissue has been studied by comparing the cellular composition of hypertonic saline-induced sputum, with bronchoalveolar lavage fluid (BAL) and bronchial mucosal biopsies. One such study compared induced sputum with bronchoscopic bronchial washing and BAL in healthy volunteers and asthmatics. It showed that sputum had a higher proportion of non-squamous cells and higher levels of ECP, albumin, and mucin-like glycoprotein. The eosinophil numbers and ECP levels in sputum correlated more closely with those in bronchial washing than in BAL. The proportion of eosinophils was higher in sputum and bronchial washing from asthmatic subjects compared with healthy volunteers [75]. In mild to moderate atopic asthmatics the percentage of eosinophils in sputum was significantly correlated with that in bronchial wash and in BAL, whilst there was a trend towards such a correlation between the number of eosinophils in sputum and the number of

EG₂⁺ eosinophils in bronchial biopsies. In addition, the proportion of CD₄⁺ lymphocytes correlates between sputum and BAL [76, 77]. Another group has shown induced sputum to be relatively rich in eosinophils and neutrophils, but with less lymphocytes and macrophages compared with bronchial washings and BAL fluid [78].

The future

Provided sputum is induced and processed in a standardized manner, it provides a safe and reliable assessment of airway inflammation. However, interpretation of results obtained in different studies is hindered by differences in the techniques of sputum induction and processing. For clinical trials it will be important to further elucidate the clinical significance of changes in sputum composition. Improvements and standardization of sputum methodology will increase its use in clinical practice as well as increasing the number of research applications of sputum. This will allow more specialized techniques to be applied, such as flow cytometry, *in situ* hybridization, polymerase chain reaction, as well as immunocytochemistry and cell culture.

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E. M. ERIN*, P. J. BARNES† and T. T. HANSEL*

**Clinical Studies Unit and*

†Department of Thoracic Medicine

National Heart and Lung Institute

Imperial College

London SW3 6HP

UK

E-mail: t.hansel@ic.ac.uk