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Sputum induction: a method to assess airway inflammation in asthma

A. Spanevello*, G.B. Migliori*, A. Satta*, M. Neri*, P.W. Ind**

*Dept of Pneumology, Fondazione Clinica del Lavoro IRCCS, Tradate, Italy. **Respiratory Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, London, UK.

Correspondence: A. Spanevello, Fondazione Clinica del Lavoro, Centro Medico, via Roncaccio 16, 21043 Tradate (VA), Italy.

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ABSTRACT

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In recent studies, sputum cell counts have been used to examine the cell and molecular markers of airway inflammation. In this paper, we describe three different technical methods of analysing sputum samples: the first using smears, the second using cytocentrifugation after selection of a mucous plug, and the third using cytocentrifugation to analyse the entire sputum sample.

These last two techniques have been used in a pilot study to compare the differential cell counts in sputum and bronchoalveolar lavage (BAL). The results show a significant correlation between the percentage of the eosinophils in sputum and bronchial sample of the BAL.

Previous results on the study of airway inflammation with the analysis of sputum and the preliminary data on the relationship between sputum and BAL confirm the usefulness of this noninvasive technique in the understanding of the pathogenesis of asthma.

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Airway inflammation plays a major role in the pathogenesis of asthma. Epithelial damage, mucus production, and mast cell and eosinophil infiltration are characteristic features [1, 2]. A simple, convenient method of directly quantifying airway inflammation would be of great value in improving the understanding of the pathogenesis of asthma.

Airway inflammation has been studied by bronchoalveolar lavage (BAL) and bronchial biopsies [3, 4]. Recent studies have emphasized the safety aspects of fibreoptic bronchoscopy and endobronchial biopsy in asthma [5, 6]. However, BAL and bronchial biopsies create discomfort in patients, and cannot easily be applied repeatedly over short periods of time to follow serial changes in airway inflammation during exacerbations or the effects of a treatment in large groups of patients.

Recently, sputum has been used to examine the cell and molecular markers of inflammation, and several methods have been described [7]. In this paper, we describe the different technical approaches to the analysis of sputum cells and mediators and present preliminary data on the relationship between the differential cell count in induced sputum and BAL of asthmatics.

Methods of sputum examination

Method using smears after selection of mucous plug

Initial examination involves measurement of sputum weight and transfer of the sample to a Petri dish, where its macroscopic characteristics are recorded [8, 9]. The quality of the sample was assessed estimating the volume of lower respiratory tract secretions (size and number of plugs) and the degree of salivary contamination [9].

A portion of each plug was placed in trypsin [8] or Sputalysin (Calbiochem, San Diego, California, USA) [9] and incubated for 2 h at 37°C. A total cell count was performed using an haemocytometer, after which each plug was smeared on slides and air dried.

Differential cell counts were performed by counting 400 nucleated cells on each of two slides fixed with methanol and stained with May-Grünwald Giemsa. Two further slides were fixed with Carnoy's solution and stained with 0.5% toluidine blue in 0.7 N hydrochloric acid (pH 0.1), and 1,500 cells were counted on each to determine percentage counts of metachromatic cells.

Method using cytocentrifugation after selection of mucus plug

The sputum was collected in a sterile container and examined within 2 h. It was poured into a Petri dish and examined under an inverted microscope to select the portions of the sputum with little or no squamous cells. The weight of the sputum was recorded, dithiothreitol was added and mixed with the sputum by aspiration in and out of a pipette about 20 times [10], or incubated at 37°C for 20 min to ensure homogeneity [11]. The sample was washed with phosphate-buffered saline (PBS), filtered through nylon gauze, and the total cell count was measured using an haemocytometer.

The filtered cell suspension was diluted with PBS and placed in a cytocentrifuge; cytospins were prepared at 450 rpm for 6 min. Separate cytospin slides were fixed by methanol, formalin, Carnoy's solution and periodate-lysineparaformaldehyde (PLP) and stained, respectively, by May-Grünwald Giemsa for differential cell counts, by chromotrope 2R for eosinophils, by toluidine blue for metachromatic cells, and using immunochemistry techniques [10, 12, 13].

Method using cytocentrifugation to analyse the entire sputum sample

The entire sample produced was collected, the volume of sputum and saliva was determined and an equal volume of dithiothreitol was added. The samples were mixed using a vortex mixer and placed in a water bath at 37°C to ensure homogenization. The samples were removed from the water periodically for further brief vortex mixing. Aliquots of the homogenized samples were used to determine the total cell count using an haemocytometer. The remainder of the homogenized sputum and saliva was centrifuged, and the supernatants were frozen and stored at -70 °C for later biochemical analysis. The cell pellets were resuspended in saline and cytocentrifuged and stained with Diff-Quik. At least 200 nonsqamous cells on each sputum slide and at least 400 cells on each saliva slide were counted [14].

Relationship between sputum and other techniques to assess airway inflammation

We are not aware of published comparisons of the relationship between sputum and bronchial biopsies in the assessment of airway inflammation. However, FAHY *et al.* [15] have published preliminary results comparing markers of inflammation in induced sputum, bronchial wash, and BAL from healthy and asthmatic subjects [15]. They reported that the percentage of eosinophils was highest in entire samples of sputum [14], and lowest in secretions collected by BAL in asthmatics. Within individual asthmatics, the percentage of eosinophils and the concentration of eosinophil cationic protein (ECP) in the sputum correlated more closely with the percentage of eosinophils and ECP level of bronchial wash than with BAL.

In a preliminary study [16], we have evaluated the relationship between induced sputum and BAL in six asthmatic patients (table 1). Patients were excluded from the study if they had taken inhaled or systemic corticosteroids or nedocromil sodium during the month before the study; treatment with β_2-agonists was withheld for only 8 h. Sputum induction was performed as described by PIN *et al.* [9], and the analysis of the sputum as described by MAESTRELLI *et al.* [11], *i.e.* staining the slides with May-Grünwald Giemsa for differential cell counts of leucocytes, squamous and bronchial epithelial cells. BAL (3×50 mL aliquots of saline at 37°C) was performed after inhalation of 200 µg of salbutamol, as was sputum induction. Cell differential counts were performed after cytocentrifugation and staining with May-Grünwald Giemsa. We considered the recovery of the first aliquot to be a bronchial sample [17]. The preliminary data (table 2) showed a significant correlation (nonparametric rank correlation coefficient test: r=0.94; p<0.05) between the percentage of eosinophils in the sputum and in the bronchial sample.

Table 1. – Characteristics of asthmatic patients

Pt No.	Age yrs	Sex	Atopy+	Baseline FEV1 % pred	Post BD* FEV1 % pred	Aas† score
1	60	M	Yes	88	101	2
2	41	F	Yes	93	107	1
3	34	M	Yes	71	98	2
4	48	M	No	96	105	1
5	51	M	Yes	55	78	4
6	66	F	Yes	86	102	3
Mean	50			81	99	
SD	12			14	11	

+: patients were considered to be atopic if they had a positive skin-prick test to at least one common allergen extract; *: 20 min after salbutamol inhalation (200 µg). Pt: patient; FEV1 forced expiratory volume in one second; % pred: percentage of predicted; BD: bronchodilator; M: male; F: female. †: score of clinical severity of asthma (1–5).

Table 2. – Total and differential cell count of induced sputum (SI), bronchial sample (BS) and alveolar sample (AS) in asthmatics

		SI	BS	AS
Total cells		11.4±5.4 $\times10^3\cdot mg^{-1}$+	13.0±2.7 $\times10^4\cdot mL^{-1}$	25.8±9.0 $\times10^4\cdot mL^{-1}$
Macrophages	%	51.4±11.40	73.6±6.68	84.6±3.58
Neutrophils	%	27.8±11.60	8.6±3.62	4.6±2.10
Eosinophils	%	9.7±8.03	5.9±6.49**	2.0±0.18
Lymphocytes	%	1.0±0.62	2.6±1.53	7.0±1.81
Epithelial cells	%	3.3±0.99	6.9±5.05	2.0±1.62
Squamous cells	%	6.7±1.77	2.3±1.95	0.7±1.62

Data are presented as mean±SD. +: weight of sputum portion (expressed as mean±SD) = 53.3±13.4 mg. **: significant correlation between percentage of eosinophils in sputum and bronchial sample (Spearman rank test: r=0.94; p<0.05).

Discussion

In this paper we have described the different approaches which have been used to analyse sputum cells and mediators, and we have presented preliminary data on the relationship between the differential cell count in induced sputum and BAL of asthmatics.

We believe that it is important to emphasize three points. Firstly, some authors have obtained results in analysis of induced sputum similar to those seen in other studies using BAL in asthmatics. PIN *et al.* [9] showed that eosinophils and metachromatic cell counts were higher in sputum from asthmatics than healthy subjects, and the differences were similar to those found in BAL. They also showed that there was an increase in eosinophils and metachromatic cells 24 h after allergen inhalation, causing early and late asthmatic responses and heightened methacholine airway responsiveness [18]. FAHY *et al.* [19] also performed sputum induction before and after aerosolized allergen challenge. They found that the median percentage of eosinophils and neutrophils in induced sputum samples was significantly higher 4 and 24 h after allergen challenge than at baseline.

The airway inflammatory response to allergen inhalation had been previously studied by BAL, and it was characterized by an influx in neutrophils and eosinophils [20] similar to those seen in sputum of asthmatics.

MAESTRELLI *et al.* [11] indicated that isocyanate-induced asthmatic responses are associated with an influx of eosinophils in airway secretions. Eosinophil accumulation in the airways after specific bronchial challenge has been reported in occupational asthma using BAL [21].

Secondly, the method for the analysis of sputum using smears could be considered limited in comparison to BAL because it allows determination of cell counts only, whereas the new methods using the cytocentrifuge permit immunohistochemical staining, analysis of supernatant, and processing for flow cytometry [22]. In fact HARGREAVE *et al.* [10, 12, 23] have been able to identify the cellular expression of prostaglandin EG_2, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumour necrosis factor (TNF) and interleukin-8 (IL-8) cell activation markers in sputum. FAHY *et al.* [14] have measured fibrinogen, albumin, eosinophil cationic protein (ECP), tryptase and histamine in the supernatant of induced sputum from asthmatics, and compared the results with those from healthy subjects. WIRCHOW *et al.* [24] previously reported high levels of ECP in the supernatant of sputum from asthmatics.

Thirdly, both the preliminary data by FAHY *et al.* [15] and ourselves [16] seem to confirm that the information obtained from induced sputum and the bronchial sample of BAL are derived from similar compartments, though larger studies are required.

Analysing the points described above, we consider sputum induction to be a noninvasive alternative in the study of airway inflammation, and think that it will play an important role in understanding the pathogenesis of asthma.

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