Review

The role of induced sputum in asthma assessment

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SUMMARY. During recent years interest has been growing in the use of non-invasive methods for the assessment of airway inflammation in subjects with asthma. To date sputum induction is the only non-invasive measure of airway inflammation that has a clearly proven role in asthma management. Induced sputum cell count and mediator measurements have been particularly well validated. A variety of soluble mediators can be measured in the sputum supernatant of patients with asthma, including eosinophil-derived proteins, cytokines and remodelling-associated proteins. Sputum eosinophilia (i.e., >3%) is a classic feature of asthma, although a minority of patients present a non eosinophilic cellular pattern. The percentage of sputum eosinophils has proved to be useful in predicting short term response to inhaled corticosteroids, and there is scope for the application of other induced sputum markers in clinical practice. Sputum induction is a procedure that is generally well-tolerated and safe and a European Respiratory Society (ERS) Task Force has published a comprehensive review on sputum methodology. The widespread application of induced sputum in the investigation of asthma across the complete spectrum of disease severity, and mainly in moderate to severe asthma, has provided insight into the relationship between airway function and airway inflammation leading to the proposal of new disease phenotypes and the definition of which of these phenotypes respond to current treatment, offering an additional tool to guide the clinical management of patients with asthma. *Pneumon 2012, 25(3):283-287.*

INTRODUCTION

Asthma is a chronic airways disease characterized by inflammation, hyperresponsiveness and structural changes of the airway wall, known as remodelling, all of which lead to chronic airflow obstruction and decline in lung function[1]. Various different types of cells and inflammatory mediators have been shown to participate in the above processes. Asthma is considered to be a complex disease with a variety of triggers, different

responses to treatment and different types of inflammation. Medical research has therefore been focused on possible biomarkers which may better express both the type of inflammation and the response to treatment.

The use of invasive methods such as fiberoptic bronchoscopy, in conjunction with bronchial biopsy and bronchoalveolar lavage (BAL), has partially elucidated the role of the different types of inflammatory cells in asthma, such as eosinophils, mast cells and T-cells and, more recently, the structural cells of the airways, such as fibroblasts, endothelial cells and epithelial cells[2]. Bronchoscopy, however, is of limited use in routine clinical practice due to its invasive nature, and it is not suitable for the regular monitoring and investigation of response to treatment of a chronic disease such as asthma. During recent years, the application of semi-invasive techniques has tended to overcome this disadvantage. Sputum induction has inaugurated a new era in asthma research and management. Although it has been known for a long time that sputum spontaneously produced by individuals with asthma contains more eosinophils than that of healthy subjects, it is only in the last two decades that sputum induction has gained wide application, with definition of algorithms for its use. After the first description and standardization of the method in 1992 by Pin et al[3], an impressively large number of papers have been published, culminating in the presentation of a comprehensive review on sputum methodology by the ERS Task Force[2,4,5].

METHODOLOGY OF SPUTUM INDUCTION

The aim of sputum induction is the collection of an adequate sample of secretions from the airways from patients who cannot produce sputum spontaneously. Theoretically, sputum collected by this method comes from the more peripheral airways and therefore its value may be greater. Induced sputum provides information about the cellular and molecular components of airway inflammation. The inhalation of aerosolized hypertonic or hypotonic saline can cause production of sputum which can be collected and analyzed. The mechanisms of sputum induction are not fully understood but it is thought that the high osmolarity of the fluid deposited in the airways augments the vasopermeability in the bronchial mucosa and results in the production of secretions by the submucosal glands.

Sputum Collection

Induced sputum must be collected early in the morning. The patient inhales hypertonic saline with the use of an ultrasonic nebulizer in two different ways:

a) inhalation of the same (3%–4.5%) or increasing (3%, 4% and 5%) concentrations of aerosolized hypertonic saline over fixed time periods[3,6]

b) inhalation of the same concentration of hypertonic saline (4.5%) over increasing time periods[7].

The choice of technique appears to influence neither the total nor the differential sputum cell count. The duration of sputum induction should be kept standard and usually ranges from 10-20 minutes. The patient undergoing the shorter time induction (5 minutes) gives a sample with more granulocytes (eosinophils and neutrophils) than the patient undergoing the longer time induction, whose sample contains more mononuclear cells[8]. The concentration of mucus is higher in samples collected earlier (0-4 minutes) than in those collected at a later time point (16-20 minutes), and the converse applies for the concentration of surfactant protein. This is indicative of the fact that the material collected at different time points during sputum induction originates from different parts of the respiratory system. Thus, samples collected earlier in the process may represent the biofluid from the central airways, while those collected later possibly contain the biofluid from the more peripheral airways and alveoli[8,9].

It is important to establish the total volume of the inhalation saline required, although there is no pre-defined volume of inhalation saline for the induction of an efficient sputum sample. It is not yet known whether induction is better achieved by changing the duration of inhalation or the output of the nebulizer or both[10]. Finally, the size of the various corpuscles influences both their anchorage (fixation) and distribution (mode) in the airways, which could also affect the sputum composition and induction effectiveness[11]. The effect of the nebulizer accessories (length of pipes, valve, etc.) has not been systematically evaluated, but there is agreement about the use of an ultrasonic nebulizer with an output of ~1ml/min for 15 to 20 minutes in order to achieve high probability of an adequate sputum sample[12].

As inhalation of hypertonic saline may result in some degree of bronchoconstriction, it is recommended for patients to be pretreated with inhaled short acting β_2 agonists before sputum induction[13]. The application of isotonic rather than hypertonic saline in patients with post bronchodilator FEV_1 <65% and the addition of a short acting β_2 agonist (salbutamol) in the cup of the nebulizer with hypertonic saline during sputum induction has been suggested, without this influencing the total cell count

of the induced sputum[14,15].

Sputum processing and analysis

Once a sputum sample has been obtained sputum processing should be initiated within two hours in order to avoid significant changes in the number of cells and inflammatory mediators. Alternatively, placing the sample in the refrigerator at 4ºC allows delay of its processing for up to 9 hours after the collection[16].

The most crucial step of the analytical method is the selection of the sample. Three different techniques of processing have been proposed: i) processing the entire expectoration specimen given by the patient without selection, ii) selecting all the viscid or denser portion taken from the expectorated sample, which effectively minimizes contamination with saliva, and iii) use of an inverted microscope for better exclusion of saliva and its epithelial cells (this is in practice a modification of technique (ii) in which selection relies on the eye of the investigator)[4]. After selection, the sample is put into a polystyrene tube which has been preweighed empty. The next step is the weighing of the tube with the sample, followed by addition of a quantity of 0.1% Dithiothreitol (DTT) solution or its equivalent Dithioerythritol (DTE) × 4 volumes of the weight of the sample, to break the disulphide bonds in the mucin molecules, allowing release of cells[17]. Subsequently, the sample is agitated on a vortex mixer for about 30 seconds and then placed for homogenization on a tube rocker (3D-Shaker) at 22ºC for about 20 minutes. An equal volume of phosphate-buffer saline (PBS) solution is then added, to achieve a more efficient dilution of the sample and dispersion of the cells. The sample is agitated again on vortex for about 15 seconds and homogenized on the tube rocker for about 5 minutes. Sample filtration through a 48 μm nylon mesh is strongly recommended for the removal of remaining mucus and debris. This filtration process, along with the following centrifugation, slightly reduces the total cell count (TCC)[18]. Centrifugation at 300-1500×g is continued for about 10 minutes and the emerging supernatant is stored at -70ºC. The sediment is used for the estimation of TCC with the use of a haemocytometer (Neubauer) and trypan blue staining. The cell pellet is diluted with PBS to an adjusted concentration of 1.0×10^6 cells/ml. Finally, cytospin smears are prepared with cytotubes (40-65 μl of the sample in each) and placed for cytocentrifugation at 22×g for 6 minutes[19]. The smears are stained with Giemsa and May-Grunwald stains, in order to determine the differential cell count (DCC), counting a minimum of 400 nonsquamous cells, and the result is reported as the percentages of macrophages, neutrophils, lymphocytes, eosinophilis and bronchial epithelial cells, among the total nonsquamous cells. The percentage of squamous cells should always be reported separately as a marker of sample suitability (it should be less than 20%)[4].

SPUTUM CELL COUNTS

In patients with asthma, induced sputum contains more living cells than spontaneously produced sputum[20]. Higher than normal eosinophil counts are observed in patients with symptomatic asthma[21,22]. Up to 80% of patients with asthma never treated with inhaled or oral corticosteroids and up to 50% of those who are under such medication, appear to have sputum eosinophilia, which is usually defined as a sputum eosinophil count of greater than 3%[23,24]. Patients who are experiencing an asthma attack usually have sputum eosinophilia, although some patients under corticosteroid treatment may show a high sputum neutrophil count during an exacerbation[24]. Non-eosinophilic (mainly neutrophilic) exacerbations of bronchial asthma have been described based on the prevailing cell count, but neither their cause nor their frequency is known. A possible cause of this might be viral infection[25,26]. The validity of a high sputum eosinophil count has been judged to be better than peak expiratory flow (PEF) variability for the identification of asthma, while the acute bronchodilator response has been evaluated in a study to have sensitivity and specificity comparable to those of airway responsiveness[27]. There is poor association, however, between asthma severity, airway responsiveness and sputum eosinophilia[22,28], although one study showed an inverse ratio between sputum eosinophil count and clinical symptoms, FEV_1 or PC_{20} methacholine[21].

Generally, asthma can be categorized into four subgroups based on the prevailing sputum cell count, namely: eosinophilic asthma (>3% eosinophilis), neutrophilic asthma (>60% neutrophils), mixed granulocytic asthma (>3% eosinophils and >60% neutrophils) and paucigranulocytic asthma (<3% eosinophils and <60% neutrophils)[29].

Categorization of patients with asthma according to sputum eosinophilia is of great use in those with symptoms who are treated with inhaled corticosteroids, because additional therapeutic choices (e.g., long acting β_2 agonists, higher dosage of inhaled corticosteroids, leukotriene receptor antagonists and theophylline) differ in their effects on eosinophilic airway inflammation[30,31]. Clinical improvement and a reduction in sputum eosinophil

count have been shown in patients with asthma taking montelukast for 4 weeks[32].

The short-term response to inhaled corticosteroids differs significantly according to the sputum eosinophil count. There is evidence that patients with < 3% sputum eosinophils show little improvement in their symptoms, suggesting that assessment of the underlying airway inflammation might provide a more reliable guide to the need for corticosteroids than functional abnormality (FEV_1). Further studies are needed to define the relationship between airway inflammation, symptoms and the response to corticosteroids[12].

It should be acknowledged that some patients show persistent eosinophilic airway inflammation despite corticosteroid treatment and whether this group of patients could be improved with an increase of anti-inflammatory medication is a pertinent question. Documentation provides support for the hypothesis that persistent sputum eosinophilia in patients with "severe asthma" may be attributed to inadequate treatment or poor compliance with inhaled corticosteroid therapy[33].

Sputum neutrophilia appears to be associated with more severe disease[34]. Both the neutrophils percentage and the absolute sputum neutrophil count have been correlated with low post bronchodilator FEV_1, lending support to the hypothesis that neutrophilic airway inflammation may play a role in the progression of persistent airflow limitation in asthma[35]. It is possible that some patients with non-eosinophilic asthma treated with inhaled corticosteroids have residual eosinophilic inflammation in their airway walls. In addition, it has been suggested that in such patients the presence of macrophages with large red areas may predict a relapse of eosinophilic airway inflammation when they are weaned off the use of inhaled corticosteroids[36].

Recent interest has been directed towards investigation of the relationship between female hormones and obesity in asthma and it has been shown that inflammation in sputum is neutrophilic in menopausal women and eosinophilic in premenopausal women[37]. Although obese patients with asthma appear to have poorer control of their disease than non obese patients with asthma, no correlation has been demonstrated between body mass index (BMI) and sputum inflammatory cell count[38].

The value of the assessment of inflammatory markers in the induced sputum of patients with occupational asthma has been investigated[39,40]. Generally, occupational asthma is characterized by findings in sputum similar to those in non occupational asthma, and specific challenge testing with occupational allergenic agents is accompanied by increase in the sputum eosinophil count[39-41]. There is evidence that the eosinophil count is increased during workplace exposure in patients with occupational asthma and reduced after their removal from the work environment[40,42]. Sputum eosinophilia appears to predict a more severe stage of asthma and greater bronchodilator reversibility but is not related with PEF in work exposure[43]. It has been reported that subjects with occupational asthma may show sputum neutrophilic inflammation[44].

SPUTUM SUPERNATANT ANALYSIS

Inflammatory mediators in sputum supernatant have been studied in relation to asthma and its progression. Various different aspects of inflammation and remodelling may be reflected by these mediators, including activation of eosinophils [e.g., eosinophil cationic protein (ECP)], activation of mast cells (e.g., tryptase), cytokine production [e.g., interleukin-5 (IL-5), tumour necrosis factor-α, (TNF-α)] and augmentation in vascular permeability (e.g., albumin, fibrinogen)[23].

DTT causes fission of disulfide bonds and denaturation of proteins, which is why its use has been considered to have an effect on cellular markers that are protein molecules. The use of DTT does not appear to affect the concentration of mediators such as IL-5, tryptase, histamine and immunoglobulin A (Ig-A)[45]. In contrast, it has been suggested that the protease inhibition achieved with the use of DTT aids in the detection of cytokines and chemokines[46].

Asthma classification into different phenotypes has been aided by the measurement of various biomarkers in sputum supernatant. More specifically, among patients with severe asthma, those with frequent exacerbations were characterized by higher levels of IL-5 and GM-CSF compared to those with persistent airway obstruction[47].

Sputum supernatant analysis may also contribute to the evaluation of asthma treatment. Cysteinyl-leukotrienes have been detected in the sputum supernatant of patients with asthma and it is reported that the use of montelukast (a leukotriene receptor antagonist) inhibits the eosinophilic chemotactic activity in both corticosteroid-naïve and corticosteroid-treated patients[48]. Serial measurement of inflammatory mediators such as GM-CSF, RANTES and IL-8 in cells collected in induced sputum and cultured with beclomethasone dipropionate (BPD), salbutamol and formoterol, supports the benefit of using a combination of beclomethasone with β_2-agonists over beclomethasone

alone in asthma treatment, revealing a decrease in the levels of these mediators[49].

The supernatant of induced sputum has made a fundamental contribution to comprehension of the pathophysiology of asthma, and specifically the inflammation that characterizes this disease. In a recent study of subjects with mild to moderate asthma in stable condition, cytokine production by sputum cells was compared in patients with eosinophilic and non-eosinophilic inflammation. Those with eosinophilic inflammation had higher levels of IL-4 and TNF-α than healthy subjects[50]. In another study, induced sputum from patients with moderate to severe asthma treated with inhaled corticosteroids had higher levels of IL-6 than that from healthy subjects, reflecting impaired innate immunity[51].

Induced sputum pH appears to reflect an aspect of asthma other than eosinophilic inflammation, as it is related to different pathophysiological features. Patients with asthma, both controlled and uncontrolled, have lower induced sputum pH levels than healthy subjects[52]. There are also reports of greater oxidative stress in the airways of patients with refractory asthma, who showed increased xanthine oxidase and 3-nitrotyrosine activity in sputum compared with patients whose asthma was well controlled[53].

Apart from chronic inflammation asthma exhibits the additional and equally fundamental feature of airway remodelling. Many mediators have been linked to the remodelling process, including procollagen synthesis peptides, matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs)[54-58], and cytokines such as tumour growth factor β (TGF-β)[59]. Mattos and co-workers showed that the induced sputum level of MMP-9 was elevated in patients with severe asthma after specific allergen challenge and that this was not affected by treatment with inhaled corticosteroids[58]. They also observed subversion in the balance of MMP-9/TIMP-1 levels, which was thought to be the cause of excessive degradation of extracellular matrix (ECM) proteins that participate in the injury–repair process[58].

Nomura and co-workers hypothesized that a change in the balance between collagen type I synthesis and degradation towards increased synthesis might be observed during exacerbations of asthma, leading to airway remodelling[59]. Similarly, it was suggested that asthma might be associated with promotion of angiogenesis, and accordingly levels of vascular endothelial growth factor (VEGF) were found elevated in the induced sputum supernatant of patients with asthma[60]. Finally, osteopontin, a glycoprotein that can function both as an ECM molecule and a cytokine, was investigated as a potential mediator in airway inflammation and remodelling in patients with asthma. A recent study demonstrated that patients with severe refractory asthma had significantly higher levels of osteopontin than those with milder forms of the disease, with a significant association between this glycoprotein and TGF-β1, IL-13 and cysteinyl leukotrienes[61].

INDUCED SPUTUM - EXHALED NITRIC OXIDE – EXHALED BREATH CONDENSATE

The fraction of exhaled nitric oxide (FeNO) and exhaled breath condensate (EBC) are two additional non-invasive techniques of airway inflammation assessment.

High levels of FeNO (>42ppb) have been correlated with eosinophilia in induced sputum of patients with asthma, but other factors, such as high dose inhaled corticosteroids, atopy and smoking, may modify FeNO levels. In a recent study, patients receiving high doses of inhaled corticosteroids (>1,000 μg fluticasone/day) had a FeNO threshold of 27 ppb and those who were current smokers a threshold of 28 ppb, while the best threshold in non atopic patients was 30 ppb for the identification of eosinophilic sputum profile[62]. In a study of patients with severe refractory asthma, it was shown that elevated levels of FeNO could significantly predict eosinophilia in induced sputum, irrespective of treatment with high dose inhaled corticosteroids; FeNO levels >19 ppb predicted a sputum eosinophil count >3% and FeNO levels <19 ppb predicted the presence of >40% neutrophils in induced sputum, irrespective of the presence of eosinophils. These findings may be explained by the resistance to corticosteroids often observed in patients with severe asthma or the persisting activation of epithelial cells, which are the main source of NO[63].

Concerning EBC pH, there is not yet enough evidence to support an association with the induced sputum cellular profile. Specifically, even though low levels of EBC pH have been correlated with eosinophilia in asthma, this does not appear to apply to patients with severe asthma. In patients with moderate asthma who showed an eosinophilic prevalence, sputum EBC pH tended to be a good predictive marker of eosinophilia[63].

EBC has also been used for measuring inflammatory mediators, although it is not easy to make direct comparison with the measurements ininduced sputum supernatant because of the wide disparity observed; for example, EBC levels of total protein and surfactant protein

A (SPA) were at least 100-fold lower than those measured in induced sputum[64].

In patients with asthma in stable condition who were active smokers, a negative correlation was reported between EBC pH and the presence of both of eosinophils and neutrophils. On the other hand, levels of FeNO higher than 14 ppb could identify with a high specificity the presence of an eosinophilic pattern in induced sputum, while FeNO levels ≤14ppb and EBC pH levels >7.20, could predict the paucigranulocytic induced sputum inflammatory pattern in smokers with asthma[65].

CONCLUSION

The growing interest in the use of induced sputum during recent years has led to a better understanding and more effective monitoring of airway inflammation in patients with asthma. Induced sputum is currently the most robust method for the routine assessment of airway inflammation and it is a procedure that is generally safe and well tolerated by most patients. Its widespread application has provided insight into the relationship between lung function and airway inflammation and has enabled the identification of new disease phenotypes and the definition of which of these respond to current forms of treatment. Sputum induction should be available in specialist centres in order for patients with severe or difficult-to-treat asthma to be evaluated and managed, as it appears to be more valuable for such patients. To date sputum induction is the only non-invasive method of airway inflammation assessment that has a proven role in the management of moderate-to-severe asthma.

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