Induced Sputum Role in Severe Asthma Phenotyping

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Abstract

Induced sputum is a method which, by inhaling hypertonic saline, provoke a person to expectorate certain amount of bronchial secretion. Sputum sample obtained consists of liquid and celullar phase. It is used over the years to investigate airway inflammation in patients with asthma and chronic obstructive pulmonary disease (COPD). Results of cell counts in induced sputum is essential in differentiation of airway eosinophilia or neutrophilia or both, or a determination of paucigranulocytic phenotype. Obtained severe asthma phenotype due to airway inflammation help us to select biological therapy and predict responders.

The correlation between "noninvasive" measures such as blood eosinophilia, or fraction of exhaled nitric oxide (FeNO) with sputum eosinophils is suboptimal. Induced sputum is a good and reproducible discriminator for eosinophilic asthma. There are some safety concerns as induced sputum procedure can provoke adverse effects such as bronchoconstriction and dyspnea, which is reversible to standardized therapy. Sputum induction is safe and well tolerated by patients with severe asthma, which supports its use in clinical and research practice.

Keywords: severe asthma, sputum induction, asthma phenotypes, safety

Introduction

Asthma is a chronic inflammatory disease of the airways that causes variable airflow obstruction and bronchial hyperresponsiveness. Many cells and cellular elements play a role in the pathogenesis of asthma. Cellular quantification in sputum samples is one of the noninvasive methods of assessing asthmatic airway inflammation. Sputum induction with hypertonic saline is used frequently for investigation of airway inflammation in patients with asthma or chronic obstructive pulmonary disease (COPD). The method was introduced in asthma by Pin et al. in 1992 and has been evolving ever since[1]. As it is relatively non-invasive technique which provides repeatable and valid results[2] sputum induction quickly gained an important place in clinical practice. The correlation between "noninvasive" measures such as blood eosinophilia, or fraction of exhaled nitric oxide (FeNO) with sputum eosinophils is suboptimal, making them poor surrogate measures of airway eosinophilia[3]. Based on sputum eosinophil and neutrophil proportions in induced sputum we could categorized four inflammatory subtypes of an airway inflammation in asthma: neutrophilic asthma, eosinophilic asthma, mixed granulocytic asthma and paucigranulocytic asthma[4]. Asthma phenotypes based on different types of airway inflammation allow us to individually tailor treatment. It has been well known for decades that sputum eosinophilia can predict response

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to corticosteroid treatment, but the phenotyping of the inflammatory response in asthma has gained even greater clinical significance with the introduction of biological therapy. A great advantage of the technique is that it enables sampling of the airways in a less-invasive manner, in contrast with other methods such as bronchial biopsy, bronchial brushing and bronchoalveolar lavage, all of which require bronchoscopy. This is particularly important in the examination of patients with severe airways disease where endoscopy poses significant risk to oxygen desaturation[5]. These techniques allow sputum to be obtained from 80 to 90% of patients, which is significantly more than patients can expectorate spontaneously. It has been shown that cells in induced sputum reflect well the findings in bronchial wash and lavage samples and are more viable than in spontaneous sputum[6]. Fahy et al. studied markers of inflammation and cells in samples obtained by sputum induction, bronchial washing and lavage samples from healthy and asthmatic subjects. Concentrations of cells and inflammatory markers were higher in induced sputum samples than in bronchial washings or lavage materials. Induced sputum samples contained higher percentages of neutrophils and eosinophils, and higher concentrations of eosinophil cationic protein, albumin and mucin-like glycoprotein, probably because they were less diluted[7]. Induced sputum was first developed as a research tool, and in the meanwhile, it became a valuable clinical tool[8].

Sputum induction procedure

Induced sputum is quite a technically demanding procedure. Each sputum induction should proceed by spirometry, and patients with FEV_1 <40% of predicted, or less than 1 L, have to be excluded from induction procedure.[35] The highest FEV_1 value as well as PEF value obtained were considered as baseline and were used to calculate a relative fall in FEV_1 and PEF during the procedure. Each subject with severe asthma who is going to do the induced sputum should be premedicated with 200 μg salbutamol. Sputum induction is performed by inhalation of increasing concentrations of aerosolized saline (0.9%, 3%, 4% and 5%) through a mouthpiece without a nose clip. In our clinic we use an Omrone NE U07 ultrasonic nebulizer (Omron Healthcare Europe) with an output of 1.0 mL/min and particle size of 3.5 μm mass median diameter to generate an aerosol. Each concentration should be inhaled over 7 minutes. After each inhalation, the patient should be asked to expectorate into a container for an analysis and FEV_1 or PEF is needed to be measured again. The procedure should be interrupted if dyspnea or wheezing occurred and immediately appropriate treatment should be provided. If there is a fall in FEV_1 or PEF on measurement between different concentrations inhalation of 10-20% versus baseline, the same concentration of saline should be used in the next inhalation interval. If the fall is greater than 20%, the procedure should be terminated.[30] Patients should be instructed to interrupt the inhalation if they need to expectorate (in this case the clock was stopped and inhalation continued after expectoration) or experience dyspnea or wheezing. The volume of the induced sputum should be recorded. Sputum sample should be immediately trasferred into cytological laboratory.

Processing of induced sputum for cytological analysis and differential cell counting

It is recommended to process induced sputum a soon as possible or within 2 hours to ensure optimal cell preservation. Two different approaches can be followed for processing, entire sputum analysis or more often selected sputum plugs method. Selected sputum plugs or „fishing" method means selection of dense viscid portions of samples for analysis with minimising of saliva contamination[9,10].

It is necessary to weight sputum plugs before the homogenisation. Homogenisation is performed by the use of fresh solution of 0,1% dithiothreitol (DTT) that breaks disulphide bonds in mucin molecules and preserves cells morphology. The added DTT volume is 2-4 times of recorded weight of the plugs, tubes with mixture are placed in the shaking water bath on 37°C for 15 minutes to ensure complete homogenisation. It is necessary to stop the effect of DTT and preserve cells morphology by adding buffer solution (PBS) in a volume equal to the sputum volume plus DTT volume.[36]

Filtration of the fluid mixture through 48-52 μm nylon gauze can remove remaining debris and mucus. Next step is recording of the filtrate volume, accessing cell viability and measuring total cell count/millilitre using haemocytometer.

Dissolved induced sputum should be processed as any other liquid cytology sample in cytocentrifuge and centrifuge. Cytospin slides are usually May Grunwald-Giemsa (MGG) stained and used for cell counting. Remaining supernatant can be stored on -80°C for additional analyses[4]. Cytological examination of the slides should be performed under light microscope high power magnifications (400x, 1000x). Adequate are samples with less than 20% of squamous cells counting on 300-500 of all cells. Differential cell count (%) should be calculated on 300-500 non-squamous cells: eosinophils, neutrophils, macrophages, lymphocytes, columnar epithelial cells and mastocytes, if any. Both counts (%) should be recorded in final report[9,10]. On the basis of cell differential counts in induced sputum different inflammatory phenotypes of acute asthma can be divided in four types[4]:

- eosinophilic asthma (EA): >3% sputum eosinophils
- neutrophilic asthma (NA): >61% sputum neutrophils and <3% eosinophils
- mixed granulocytic asthma: >61% sputum neutrophils and >3% eosinophils
- paucigranulocitic asthma: <61% sputum neutrophils and <3% eosinophils

Asthma inflammatory phenotypes

Asthma inflammatory subtypes are characterized by some important clinical differences. Eosinophilic asthma is the most common phenotype. It is very prevalent in individuals with nonsevere disease, and also accounts for approximately 50% to 60% of the total severe asthma population[11]. It has classically been associated with allergic sensitization and a T2-dominant inflammatory response. Eosinophilic group is characterised by the highest degree of airway hyperresponsiveness. Woodruff et al demonstrated that the percentage of eosinophils in induced sputum was independently associated with more severe airflow obstruction and methacholine reactivity[12]. Eosinophilic phenotype of asthma mainly responds well to corticosteroid treatment. Strategies that are based on sputum examination to guide treatment decisions have been effective in improving lung function and decreasing asthma symptoms and exacerbations. Normalization of induced sputum eosinophil counts has been shown to be an effective strategy for preventing severe asthma exacerbations and hospitalizations. Sputum examination can detect an increase in airway eosinophils up to 3 months before the development of a clinical exacerbation[13]. This approach requires frequent sputum analyses and is impractical for routine clinical use in most centres. However, the ability to analyze sputum is necessary in centers dealing with more severe forms of asthma. The effectiveness of a treatment strategy based on assessment of airway inflammation was not as clear in patients with mild asthma, suggests that sputum analysis is not necessary in those with milder asthma that responds well to initial therapy[14].

Eosinophilic inflammation in the airway mucosa that persists despite the use of high doses of inhaled corticosteroids or systemic corticosteroids is recognized as a separate phenotype, severe asthma. It has been shown that eosinophilic inflammation despite vigorous antiasthma treatment is associated with remodelling of the airways, impaired lung function, and near-fatal asthma attacks[15]. In addition to the classic allergen-mediated Th2 paradigm, innate immune stimuli such as environmental factors, air pollution, weather changes, and viral infections may be capable of eliciting Th2 responses associated with eosinophilia[16]. Recruitment of eosinophils into the airway in allergic asthma is mediated by the coordinated action of cytokines and chemokines including IL-5, IL-13, eotaxins, and the adhesion molecules P-selectin and vascular cell adhesion molecule-1. IL-5 is a critical cytokine for eosinophil generation in the bone marrow, as well as eosinophil recruitment, activation, and survival[17]. The effects of IL-13 include induction of goblet cell metaplasia and increased mucus secretion, increased airway hyperreactivity, and, indirectly, trafficking of eosinophils to the site of tissue injury via chemotaxis[18]. Based on that, sputum eosinophil count provides an effective method to identify patients who will benefit from biological therapy. Drugs targeting specific Th2 cytokines, including monoclonal antibodies against IL-5 and IL-13, have shown a promising effect in the treatment of refractory eosinophilic asthma[19]. Steroid usage in severe asthma could mask the underlying eosinophilic inflammation. There are investigations showing that single sputum measures underestimate the likelihood of asthma classification as eosinophilic phenotype.37 On some occasions, like decreasing corticosteroid therapy or after antibiotic treatment, it is neccessary to repeat the induced sputum after few weeks, to see if the eosinophils demasking will appear.Asthma in most patients in reality is eosinophilic, due to results of severe asthma patient cohorts.[38]

Neutrophilic inflammation is also a common finding among adults who have persistent asthma symptoms despite inhaled corticosteroid treatment and particularly during asthma exacerbations[20]. Douwes et al. also found that only around 50% of asthma cases was associated with eosinophilic inflammation, and that in most other cases asthma was accompanied by an increase in airway neutrophils and interleukin 8 (IL-8)[21]. Increased neutrophils have been reported in subjects with severe asthma requiring intubation and sudden onset fatal asthma, indicating a role for neutrophils in the most severe forms of asthma[22,23]. The results of the study by Jatakanon et al. indicate that an increase in the number of neutrophils is associated with a greater degree of severity of symptoms[24]. Li et al. reported that a significant proportion of asthma and wheezing illness in both adults and children is associated with neutrophilic airway inflammation and that this pattern is not limited to individuals with severe symptoms[25]. Neutrophils in severe asthma were significantly increased compared with those with mild asthma and healthy controls but not when compared with those with moderate asthma[26]. This raises important and interesting questions regarding the mechanisms and consequences of neutrophilic inflammation, as well as presenting a novel and inviting therapeutic target. Neutrophilic inflammation is most frequently induced by infection or pollutant exposure [27]

The possibility of bacterial infections as a cause of the neutrophilia was evaluated by examining for intracellular bacteria, which is a validated technique that correlates with quantitative microbiology in the detection of respiratory tract infections. Interestingly, intracellular bacteria levels were higher in asthma compared with healthy controls, which may indicate increased exposure of the lower airways to bacteria in asthma. This may

be a consequence of airway impairment and impaired local defence caused by airway mucosal damage in asthma[28].

Induced sputum analysis in research and clinical practice

Sputum induction analysis is non-invasive, but quite a technically demanding procedure. Besides, it has safety concerns, as inhaled hypertonic saline can cause adverse effects such as coughing, vomiting, bronchoconstriction and lung hyperinflation[29]. The mechanism of the effect is unknown but may involve the activation of airway mast cells or sensory nerve endings. This makes the examination uncomfortable for some patient, and monitoring of lung function during the procedure is necessary. The safety of the method was thoroughly assessed in patients with airway obstruction characteristic for asthma[29,30] and COPD[31,32]. Pretreatment with beta-agonists is a routine part of the procedure and is intended to prevent bronchospasm. In our practice some subjects reported adverse events, mostly dyspnea followed by drop in lung function, but after bronchodilator and corticosteroid therapy all recovered. During the sputum induction procedure, the patient must be carefully monitored by the medical staff, patients should be encouraged to expectorate quality and sufficient sample for cytological analysis, for which the procedure is rather demanding and complicated. That makes this procedure time-consuming. For these reasons, it is performed only in specialized respiratory clinics and as a part of research projects.

Induced sputum analysis has brought new insights into innate and adaptive immunity processes in airways, but did not answer all questions[33]. It also allowed investigations in transcriptomics, proteomics, and genomics, which are in progress, hoping to elucidate more about the complexity of inflammation[34].

Conclusion

Induced sputum is a valuable tool for determining the asthma inflammatory phenotypes. Monitoring of airway inflammation provide additional data which enables individual adjustment of treatment to each patient. With the introduction of biological therapy, precise immunological phenotyping becomes even more significant. For this reason, we would recommend that every severe asthma center should be familiar with this method and its use in clinical practice.

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