Acta clin Croat 2002; 41:89-93 Original Scientific Paper

INDUCED SPUTUM – A METHOD FOR CYTOLOGIC ANALYSIS OF BRONCHIAL SPECIMENS

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SUMMARY – Sputum induction by inhalation of hypertonic saline is a noninvasive method to obtain secretions from the lower respiratory tract for cell counting and bacteriologic analyses. The aim of this study was to introduce the method of induced sputum in our clinical practice and to get an insight into airway inflammation by examining cell count differences in 15 asthmatic patients, 30 chronic obstructive pulmonary disease (COPD) patients and 15 healthy controls. Gradually increasing concentrations (3%, 4%, 5%) of hypertonic saline were inhaled. Subjects were encouraged to cough in order to expectorate. The quality of the sample was scored on the volume of the plugs (considered to represent lower respiratory tract secretion) and salivary contamination (proportion of squamous cells in the slides). A sample score ≥4 was considered adequate, 3 intermediate, and ≤2 inadequate. Cytologic sputum analysis was done after microscopic selection of the plugs. The results showed that inhalation of hypertonic saline is a safe method to get adequate sample for investigating various inflamatory mechanisms in lower airways. There were differences in sputum from three groups of subjects, i.e. a higher proportion of neutrophils in COPD (32%) in comparison to asthmatics (15%) and healthy controls (10%). The percentage of alveolar macrophages also differed (COPD 56.5%, asthmatics 46%, healthy 40%). Asthmatic patients had a higher proportion of eosinophils (asthmatics 18.5%, COPD 1.9%, healthy 0.06%) and metachromatic cells (asthmatics 0.3%, COPD 0.039%, healthy 0.014%).

Key words: *Sputum, cytology; Pulmonary disease – chronic obstructive, diagnosis; Asthma, diagnosis*

Introduction

The analysis of cells and mediators in the airway lining fluid from patients with pulmonary diseases has traditionally been performed in samples of spontaneously expectorated sputum or on the fluid obtained by bronchoscopy and lavage. Both sampling methods are limited in their applicability. Many subjects are unable to produce sputum spontaneously. Bronchosopy can only be employed to collect airway samples from study subjects who are well enough to tolerate it. In addition, bronchosopy cannot easily be applied repeatedly to follow the evolution of changes in airway lining fluid over short periods of time.

Inhalation of hypertonic saline to produce sputum was introduced almost four decades ago by Bickerman *et al.*[1]. However, it was only for the last 8 years that the method has been used to assess the indices of airway inflammation. The results have shown the method to be suitable, valid, reliable[2,3], and responsive[4]. The noninvasive nature of the procedure is important because repeated examinations are needed to learn more about the causes and treatment of airway diseases. The mechanism by which hypertonic saline induces sputum is not known. It may involve

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Received November 26, 2001, accepted in revised form April 22, 2002

an osmotic effect[5,6], increase in the mucociliary clearance[7,8], or stimulation of glandular secretions.

There is evidence for inflammation in chronic obstructive pulmonary disease (COPD) but aspects of the inflammation have not been studied as much as those in asthma[9]. The study of Pin *et al.* resembles most of other published analyses of induced sputum in asthmatic subjects[10]. Evidence for bronchial inflammation have been shown[11]. The aim of this study was to introduce the method of induced sputum in our clinical practice and to compare cellular characteristics of the specimens of COPD and asthma patients, and healthy controls.

Subjects and Methods

Subjects

Subject characteristics are shown in Table 1. The study population comprised 30 smokers with COPD, 15 stable asthmatics, and 15 healthy subjects. Subjects with COPD were current smokers (smoking is the main feature of COPD) with a history of productive cough on most mornings for more than 3 months/year for two consecutive years, and forced expiratory volume in first second (FEV1) <70% of the predicted value. Asthma was identified by a history of asthma symptoms and previous physician diagnosis, and objectively confirmed by metacholine airway hyperresponsiveness (PC <2 mg/ml). Healthy subjects had no history of respiratory disease, and had FEV1 >81% of the predicted value.

Study design

Study subjects attended the laboratory for 3 days within a 2-week period, at the same time of the day. On the first visit, subject characteristics were documented by a questionnaire, spirometry (Jaeger body plethysmography), and allergy skin prick tests. Then they were instructed to record peak expiratory flow (PEF) in the morning and in the evening, for 2 weeks, and to enter values in a diary. On the second and third visit, the diary was checked, spirometry was performed, and sputum was induced. If sputum could not be obtained on one of two consecutive visits, another visit was scheduled and, if unsuccessful, the subject was excluded.

Sputum processing

The reservoir of a Sonix 2000 ultrasonic nebulizer was filled with 30 ml of sterile 3% saline, and all subjects inhaled the nebulized solution for 10 min. The nebulizer generates particles with a mean mass diameter of 3.9 mm and output of 3.5 ml/min. The concentration of saline was increased at 10-min intervals from 3% through 4% to 5%. If the PEF, which was measured after each concentration, fell by >20% from the starting value, or if troublesome symptoms occurred, nebulization was discontinued. Subjects were encouraged to cough throughout the procedure, and they regularly interrupted inhalation of hypertonic saline in order to expectorate sputum into a Petri dish, where its macroscopic characteristics were recorded. Sputum was processed as soon as possible within 2 h. The quality of samples was assessed by estimating the volume of lower respiratory tract secretions and the degree of salivary contamination, as described by Pin *et al.*[12]. The quality of sputum sample was scored by: (a) visual inspection and inverted microscope examination (no plugs=0, ≤4.5x9 mm= 1, >4.5x9 mm = 2); (b) salivary contamination in total cell count defined as percentage of squamous cells among nucleated cells (>10%=0, ≤10%=1, 0%=2); and (c)

Table 1. Study subject characteristics

Characteristic	COPD n=30	Asthma n=15	Healthy subjects n=15
Male/female	22/8	8/7	8/7
Age (yrs)	49.4±5.4	34.3±3.2	37.2±3.9
Duration of disease	12.6±2.9	14.1±1.8	–
Smokers	30 (100%)	–	–
FEV_1 (% predicted)	55.6±6.5	65.5±8.5	104.5±17

Data are presented as mean±SD or n (%)
COPD=chronic obstructive pulmonary disease; FEV_1= forced expiratory volume in first second

Table 2. Differences in total and differential cell count in induced sputum

Group	Total cell count ($x10^6$/ml)	Differential cell count (% of total nucleated cells)					
		Neutrophil	Eosinophil	Macrophage	Lymphocyte	Metachromatic	Epithelial
COPD		$+	$+	$+			
(n=30)	4.1 (2.3-5.9)	32.0 (21.0-43.0)	1.9 (0.6-3.2)	56.5 (47.3-65.7)	1.23 (0.5-1.96)	0.039(0.009-0.068)	4.2(2.1-6.3)
Asthma			$*			*$	
(n=15)	3.8 (1.9-5.7)	15.0 (10.0-20.0)	18.5 (2.5-30.1)	46.0 (31.2-60.8)	0.9 (0.3-1.5)	0.3(0.001-0.6)	2.9(2.0-3.8)
Healthy							
(n=15)	3.1 (2.1-4.1)	10.0 (8.0-12.0)	0.06 (0.01-0.11)	40.0 (21.2-58.8)	1.55 (0.9-2.2)	0.014(0.005-0.023)	5.3(3.1-7.5)

Values are expressed as means; COPD=chronic obstructive pulmonary disease; $p<0.01 COPD - Asthma; *p<0.01 Asthma – Healthy; + p<0.05 COPD - Healthy

salivary contamination in differential cell count by assessing the proportion of squamous cells (too many squamous cells to permit counting=0, enough squamous cells to permit counting=1, no squamous cells=2). A sample score ≥4 was considered adequate, 3 intermediate, and ≤2 inadequate. Freshly prepared trypsin at a dilution of 1:10 in distilled water was added to the sample for homogenization. The samples were gently mixed and placed in the incubator at 37 °C for 2 hours. Ten microliters of the homogenized sputum were used to determine total cell count using a standard hemacytometer. The remainder of the homogenized sputum and saliva was centrifuged at 2000 rpm for 10 min. Cell pellets were cytocentrifuged. 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 stained with 0.5% toluidine blue in 0.7 N hydrochloric acid at pH 0.1, and 1500 nucleated cells were counted on each to obtain a differential count of metachromatic cells. The last two slides were fixed with phosphate buffer and stained with diluted Giemsa solution for eosinophil count analysis.

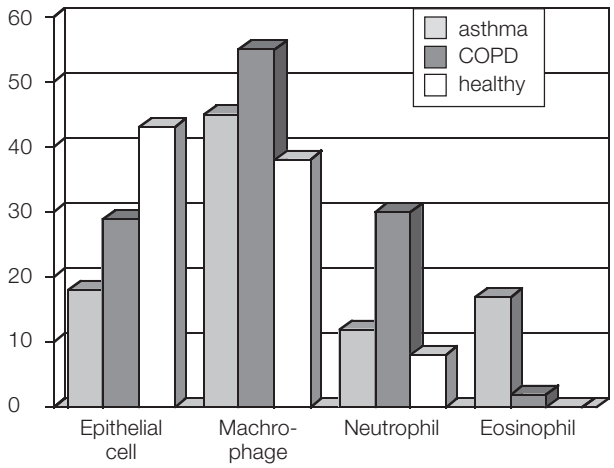

Fig. 1. Differential cell count in COPD, asthmatic and healthy subjects.

Statistical analysis

Differential and total cell counts were calculated as arithmetic means with 95% confidence intervals. Two-tailed unpaired t-tests were used to compare cell counts between groups. Significance was accepted at the 95% level.

Results

The procedure was well tolerated in 57 patients. Significant fall in PEF was observed in three patients, and the induction was stopped. Adequate samples (one sample of each concentration) were produced in 51 patients, whereas six patients produced inadequate sputum samples.

Differences in total and differential cell counts in induced sputum between study groups are shown in Table 2 and Fig. 1. There was no difference between the subjects with COPD, asthmatics and normal subjects in total cell counts, lymphocytes and bronchial epithelial cells. In comparison with asthmatics and healthy subjects, the sputum from COPD patients contained higher proportions of neutrophils (COPD 32%, asthmatics 15%, healthy 10%) and alveolar macrophages (COPD 56.5%, asthmatics 46%, healthy 40%). Asthmatic patients had signficantly higher

proportions of eosinophils (asthmatics 18.5%, COPD 1.9%, healthy 0.06%) and metachromatic cells (asthmatics 0.3%, COPD 0.039%, healthy 0.014%).

Discussion

The results of cytologic and bacteriologic analysis of bronchial secretions depend on the sputum sample quality. Inhalation of an increasing concentration of hypertonic saline enables an adequate specimen from the lower respiratory tract to obtain in a noninvasive way. There has been a concern that the inhalation by itself could alter the cellular or fluid-phase contents of sputum[13].

The aim of this study was to evaluate the safety and usefulness of the induced sputum method, and to assess the possible cytologic differences in patients with COPD and asthma.

Comparison of various sputum contents showed differences between the study groups. Asthmatics had higher proportions of eosinophils and metachromatic cells, which is comparable to the results of Pizzichini *et al.*[2]. In the COPD group, neutrophils were the predominant cells. The differential counts of macrophages were higher in COPD group than in asthmatics and healthy subjects. Similar results have been reported by Virchow *et al.*[14].

Different cells appeared to be important in the pathogenesis of airway inflammation in COPD[15]. The dominanat effector cells are neutrophils, lymphocytes and macrophages, however, eosinophils seem to be important during exacerbations of COPD. In this study, induced sputum in COPD exacerbation was not evaluated. Nevertheless, we found a greater percentage of eosinophils in COPD than in healthy controls, which may suggest the possible role of eosinophils in COPD.

The role of lymphocytes is important in bronchial mucosa, but neutrophils are dominant in the sputum and lumen of the airway[11]. This suggests that there may be mechanisms which control the traffic of inflammatory cells from the blood through the airway mucosa into the lumen. Lymphocytes may be trapped in the mucosal cells and neutrophils pass into the lumen as anti-infection cells. Gibson *et al*. have also shown that neutrophils are predominant cells in COPD and their number correlates with the degree of airflow obstruction[16].

According to our results, we conclude that inhalation of hypertonic saline is a useful, safe and noninvasive method of obtaining adequate bronchial specimen. The method enables repeated analyses in monitoring the state of airway inflammation. The results of sputum examination in asthmatic and COPD patients show differences in inflammatory cells, which suggests different pathophysiologic mechanisms and helps in differential diagnosis.

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Sažetak

INDUCIRANI SPUTUM – SUVREMENA METODA ZA CITOLOŠKU ANALIZU BRONHALNIH UZORAKA

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Inducirani sputum je metoda potpomognutog iskašljavanja putem inhaliranja rastućih koncentracija (3%, 4%, 5%) hipertonične otopine NaCl. Tim neinvazivnim načinom mogu se izbjeći invazivnije dijagnostičke metode poput bronhoskopije, a dobiti prikladni uzorci sekreta iz donjih dišnih putova. Cilj ovoga istraživanja bio je: a) uvesti metodu induciranog sputuma u našu kliničku praksu, b) ispitati njenu sigurnost i uspješnost indukcije i c) usporediti ukupan i diferencijalni broj stanica u 15 bolesnika s astmom, 30 s KOPB i 15 zdravih ispitanika. Kvaliteta sputuma procjenjivana je na temelju količine bronhalnih odljevaka (čepića koji dokazuju da se radi o sekretu iz donjeg dišnog sustava) te udjela stanica pločastog epitela (dokaz da uzorak čini slina). Bodovi ≥4 pokazuju da je uzorak primjeren, 3 da je srednje kvalitete, a bodovi ≤2 da nije prikladan za analizu. Rezultati su pokazali da je inhalacija hipertonične otopine sigurna metoda za dobivanje prikladnih uzoraka kojima se može dobiti uvid u različite upalne mehanizme kod bolesnika s opstruktivnim plućnim bolestima. U bolesnika s KOPB utvrđen je značajno veći udio neutrofila (KOPB 32%, astma 15%, zdravi 10%) i alveolarnih makrofaga (KOPB 56,5%, astma 46%, zdravi 40%) u odnosu na druge dvije ispitivane skupine. U bolesnika s astmom utvrđen je značajno veći udio eozinofila (astma 18,5%, KOPB 1,9%, zdravi 0,06%) i metakromatskih stanica (astma 0,3%, KOPB 0,039%, zdravi 0,014%).

Ključne riječi: *Sputum, citologija; Plućne bolesti – kronične opstruktivne, dijagnostika; Astma, dijagnostika*