

CHANGES IN CELLULAR AND BIOCHEMICAL PROFILES OF INDUCED SPUTUM AFTER ALLERGEN-INDUCED ASTHMATIC RESPONSE: METHOD FOR STUDYING OCCUPATIONAL ALLERGIC AIRWAY INFLAMMATION

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Abstract

Objectives: The purpose of the study was to analyze morphological and biochemical changes in induced sputum after the provocation with occupational allergens (mixture of flours and grains) in subjects with diagnosed occupational asthma. **Materials and Methods:** Subjects with occupational asthma and healthy volunteers had physical examination, skin prick tests with common and occupational allergens, and spirometry. Specific IgE against common and occupational allergens was also measured. Bronchial inflammation was characterized by the percentage of cells, levels of eosinophil cationic protein (ECP), and changes in mucosal permeability index. **Results:** There was a significant increase in the proportion of eosinophils, basophils, lymphocytes, and in the ECP level in induced sputum of occupational allergics after the specific provocation. **Conclusion:** Sputum induction is a reliable method for measuring allergen-induced airway inflammation.

Key words:

Diagnostics, Induced sputum, Occupational asthma, Specific challenge

INTRODUCTION

Airway inflammation is a characteristic feature of asthma. Exposure to occupational agents can induce eosinophilic inflammation in sensitized subjects [1].

In recent years, induced sputum has become a new research tool in diagnostics of occupational asthma, which provides a better understanding of the pathogenesis of this disease. Sputum induction by inhalation of hypertonic

saline is a non-invasive method of obtaining secretions from the lower respiratory tract for cell counting [2].

The aim of the study was to assess the applicability of induced sputum technique to the diagnosis of occupational asthma generated by exposure to flours and grains and to evaluate cellular and biochemical responses (the percentage of albumin and level of eosinophil cationic protein, ECP) in induced sputum.

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MATERIALS AND METHODS

Materials

Forty three patients with diagnosed occupational asthma and allergic rhinitis (18 bakers and 25 farmers) who had been admitted to the Department of Occupational Diseases participated in the study.

The diagnosis of occupational allergy was based on a positive history and a significant ($> 20\%$) fall of forced expiratory volume in 1 second (FEV_1) induced by occupational exposure, positive skin prick tests (SPTs) with occupational allergens and/or the presence of specific serum IgE.

At the time of the study, all subjects were clinically stable and their FEV_1 values made 70% or more of the predicted value. No subjects had the respiratory tract infection within the month preceding the study.

The control group consisted of 10 healthy subjects, who had never been occupationally exposed to grains and flours.

The study was approved by the Medical Ethical Committee and all participants gave their written consent.

Study protocol

First, the patients were challenged with placebo (potato flour) (stage I). At least seven days later, they were challenged with occupational allergens (mixture of flours and grains) (stage II).

Methods

Each subject had a medical history collected to gain information on: history of atopy, occupational exposure, respiratory symptoms, the presence of animals at home, and smoking status. This was followed by physical examination.

Skin prick tests with common and bakery allergens included: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, grass and trees I (alder, hazel, poplar, elm, willow) and II (birch, beech, oak, plane), moulds I and II (*Alternaria tenuis*, *Botrytis cinerea*, *Cladosporium herbarum*, *Culvularia lunata*, *Helminthosporium*, *Fusarium moniliforme*, *Aspergillus fumigatus*, *Mucor mucedo*,

Penicillium notatum, *Pullularia pullulans*, *Rhizopus nigricans*, *Serpula lacrymans*), feathers, and occupational allergens: mixed threshings, straw dust, hay dust, rye, oat, burley, maize, wheat and bakery dust, rye flour, maize flour, oat flour, barley flour, mixed flours, whole wheat flour, wheat flour, α -amylase (Allergopharma, Reinbek, Germany). The negative control was allergen diluent and the positive one-histamine dihydrochloride solution at 1 mg/ml. All tested sites were examined after 15 min: the wheal of 3 mm greater than negative control was considered positive.

Total serum IgE was evaluated using the Uni-CAP system (Pharmacia, Diagnostics, Uppsala, Sweden). Total IgE level higher than 100 kU/l was regarded as elevated.

The smoking status was denominated by three categories: current smokers, ex-smokers and non-smokers. The subjects who used to smoke daily and gave up smoking at least one month prior to the survey were defined as smokers. Non-smokers were those who had never smoked and passive smokers were defined as non-smokers who reported sharing a household with one or more smokers.

Specific IgE determination

The presence of specific IgE antibodies (Uni-CAP, Pharmacia Diagnostics, Uppsala, Sweden) for occupational allergens, such as: wheat flour, soya flour, α -amylase, *Sitophilus granarius* – (pax 4); wheat flour, rye flour, rice flour, barley flour – (fx 20); specific IgE antibodies (Allergopharma, Germany) for occupational allergens like wheat flour, oat flour, maize flour, sesame flour – (fx 3); and grass or corn – (gx 902) was assessed.

Bronchial provocation test, pulmonary function, and histamine challenge testing

Bronchial provocation test was performed in an inhalation chamber. At stage I, the subject was instructed to sift placebo and then at least seven days later, at stage II, to sift approximately 100 g of wheat, rye, corn, barley, and oatmeal flour and 50 gr of wheat, rye, corn, barley, and oatmeal grains. The whole challenge time was 30 min. The positive response was defined as $> 20\%$ fall in FEV_1 from the baseline.

Bronchial responses were measured by monitoring of FEV₁ before, 1, 5 and 24 h after the provocation (Vicatest 2A, Mijndhardt, the Netherlands).

Histamine challenge testing was evaluated according to Cockcroft et al. [3] on the day before the test and 24 h after the provocation.

Induced sputum and challenge procedure

First, each subject underwent spirometry and was asked to inhale for 10 min 3% hypertonic solution generated by De Vilbiss Nebulizer No. 646. Next, FEV₁ was measured to ensure the patients' safety. They were asked to rinse the mouth thoroughly with water and then with 0.9% saline, and to blow the nose to minimize contamination with post-nasal drip and saliva. Then all patients were instructed to cough and to expectorate sputum into a sterile plastic container. Samples were poured on a Petri dish and all portions, which were opaque and/or dense and looked unlike saliva, were gathered in 15 ml scaled sterile tube and treated in equal volume with 0.1% dithiotreitol (DTT, Fluka, Switzerland) in Dulbecco buffer (Sigma, USA). The portions were agitated fast for 15 s and then placed in a shaking water bath at 37°C for 15 min to ensure complete homogenization. The liquid yielded from selected portions was further diluted with Dulbecco buffer in a volume equal to the sputum plus DTT and softly balanced for 5 min at room temperature. The suspension was filtered by a sterile gauze to remove mucus and centrifuged at 1800 rpm for 10 min. The supernatant was aspirated to Eppendorf tubes and stored at -70°C for further analysis. The sediment was washed twice with the buffer with human serum albumin (HSA) (Hoechst, Behring, Germany) and each time centrifuged at 1400 rpm for 10 min. Then it was resuspended in the HSA buffer.

Total cell count (TCC) for nonsquamous cells and leukocytes in Turk stain were determined in a Burkner chamber. Cell viability was assessed by the trypan blue exclusion method. The cell pellet from cytopsin was stained with May-Grunwald-Giemsa and further counted for different cells. Induced sputum was performed before and 22 h after the challenge test.

The total protein content in the supernatant was evaluated following Lowry [4]. Albumin concentration was measured according to the "rocket" method of Laurell (the assay ranged between 20 and 200 µg/ml) [5]. The permeability index, i.e. albumin to total protein ratio, was calculated. Eosinophil cationic protein (ECP) levels were measured in induced sputum before and 22 h after the provocation using immunoenzyme assay (Uni-Cap, Pharmacia Diagnostics, Uppsala, Sweden). The limits of detection for the fluid-phase assays were 2.0 µg/l for ECP.

Statistical analysis

Cell proportions, the ECP level and albumin to total protein ratio, were compared with basal values by the paired t-test. The data are expressed as the mean ± SD. Correlations were assessed by Pearson's rank method. The differences were considered statistically significant at a value of $p < 0.05$. The calculations were performed with the Statistica 99 for Windows software program.

RESULTS

The characteristics of subjects with occupational airway allergy is presented in Table 1.

Table 1. Characteristics of study subjects with occupational airway allergy

Analyzed parameter	Patients (n = 43)
Age (mean ± SD) (years)	45.9 ± 11.2
Gender, M:F	23:20
Duration of exposure (mean ± SD) (years)	12.6 ± 7.7
Family history of atopy	8 (18.6 %)
Current smokers	20 (46.5 %)
Ex-smokers	15 (34.9 %)
Non-smoker	8 (18.6 %)
Total IgE (mean ± SD) – kU/l	89.9 ± 46.5

Pulmonary function and airway hyperreactivity

Allergen provocation caused a significant decrease in FEV₁ during early and late allergic reactions in subjects with occupational airway allergy (Table 2).

Statistically significant differences in PC₂₀ were found in this group of patients after the provocation with allergens, $p < 0.05$ (Table 3).

Table 2. FEV₁ values before and after specific challenge test with flours and grains in subjects with occupational allergy

Analyzed parameter	Subjects (n = 43)
FEV ₁ % predicted value – before (mean ± SD)	79.4 ± 12.8
FEV ₁ % predicted value 1 h after (mean ± SD)	50.2 ± 13.1*
FEV ₁ % predicted value – 5 h after (mean ± SD)	75.9 ± 10.9
FEV ₁ % predicted value – 24 h after (mean ± SD)	51.7 ± 11.2*

* p < 0.05 vs. baseline value.

Table 3. PC₂₀ before and after specific challenge test with flours and grains in subjects with occupational allergy

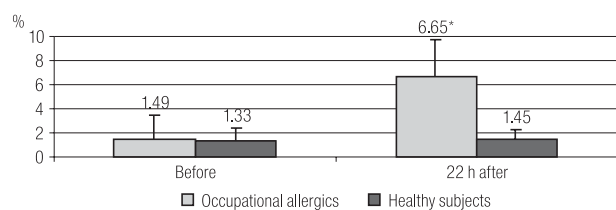
Subjects	PC ₂₀ before mg/ml	PC ₂₀ 24 h after (mean ± SD)
N = 43	3.5 ± 1.2	1.1 ± 0.6*

* p < 0.05 vs. baseline value.

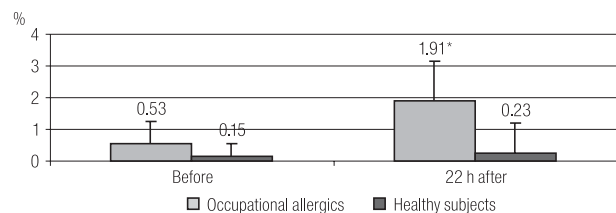
Cellular findings in induced sputum after the specific challenge test

Allergen challenge (mixture of flours and grains) caused a significant increase in the proportion of eosinophils, basophils and lymphocytes only in induced sputum of subjects with occupational asthma (Figs. 1–3).

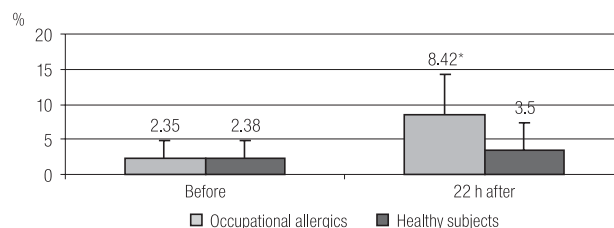
There was no significant increase in the proportion of neutrophils in induced sputum collected from occupationally allergic subjects 22 h after the specific challenge test.



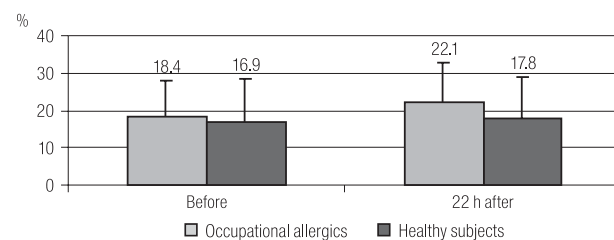
* p < 0.05 vs. baseline value.

Fig. 1. The proportion of eosinophils in induced sputum before and after inhalatory challenge with flours and grains.

* p < 0.05 vs. baseline value.

Fig. 2. The proportion of basophils in induced sputum before and after inhalatory challenge with flours and grains.

* p < 0.05 vs. baseline value.

Fig. 3. The proportion of lymphocytes in induced sputum before and after inhalatory challenge with flours and grains.**Fig. 4.** Mucosal permeability index in induced sputum before and after inhalatory challenge with flours and grains.

The specific challenge did not induce any increase in the percentage of albumin (the mucosal/vascular permeability index) 22 h after the specific challenge in induced sputum of either subjects with occupational asthma or healthy persons (Fig. 4).

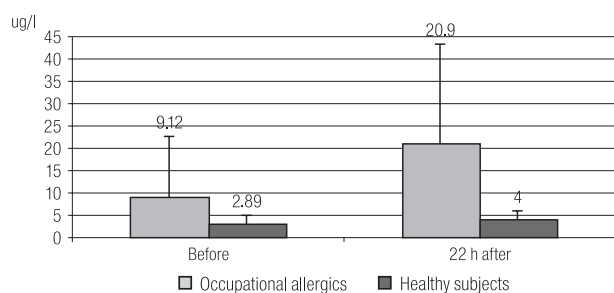
No significant changes in morphological parameters were observed in induced sputum of healthy subjects after the allergen challenge (Figs. 1–3).

Neither were observed statistically significant changes in morphological and biochemical parameters of induced sputum from patients with occupational asthma and from healthy persons after placebo challenge.

Mediator levels in induced sputum after the specific challenge test

The inhalation challenge with mixture of flours and grains induced a significant increase in the ECP level during the late phase of allergic inflammation in patients with diagnosed occupational asthma (Fig. 5).

There was a positive relationship between bronchial hyperreactivity assessed by PC₂₀ measured 24 h after the allergen challenge and both the ECP level and proportion of basophils in induced sputum of occupational al-



* $p < 0.05$ vs. baseline value.

Fig. 5. The ECP level index in induced sputum before and after inhalatory challenge with flours and grains.

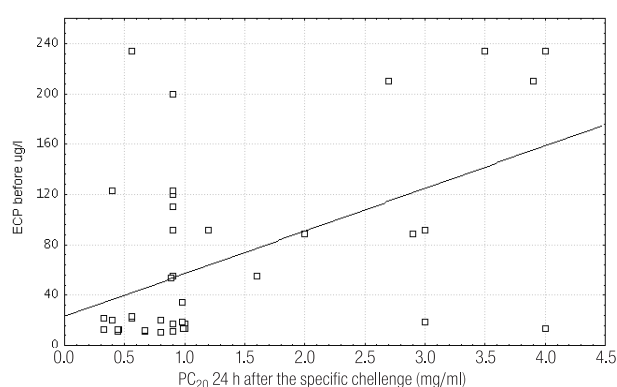


Fig. 6. Relationship between PC_{20} and ECP level.

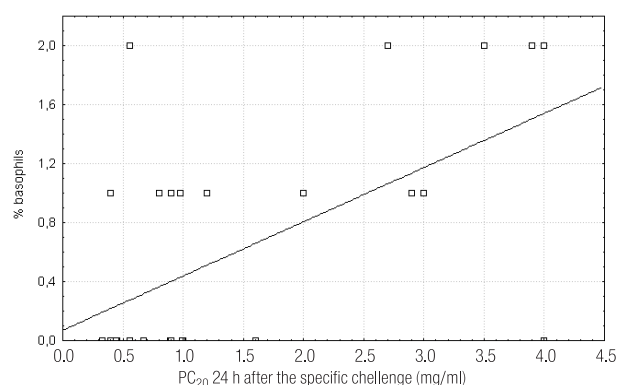


Fig. 7. Relationship between PC_{20} and the proportion of basophils.

lergics obtained before the specific provocation, $p < 0.001$ (Figs. 6 and 7).

No significant correlation was found between bronchial hyperreactivity assessed by PC_{20} measured 24 h after the allergen challenge and the proportion of lymphocytes in induced sputum of patients with diagnosed occupational asthma ($r = -0.20$, $p > 0.05$).

DISCUSSION

The most convincing way to diagnose occupational asthma is to monitor the clinical and functional status of a worker in the laboratory during and after exposure to a noxious agent. Monitoring should not only include airway calibre, but also changes in airway responsiveness to histamine as well as morphological and biochemical changes in biological fluids, e.g., induced sputum and bronchoalveolar lavage.

The use of induced sputum examination has been developed as a non-invasive research tool for objective assessment of the presence, nature and severity of airway inflammation in asthma and other respiratory diseases [2,6]. Some data demonstrated the usefulness of this method in the diagnostics of occupational asthma and other diseases [7,8]. The pathologic features of occupational asthma are similar to those of atopic asthma, as exposure to occupational agents may induce eosinophilic inflammation in occupationally sensitized subjects.

In this study, we found a significant increase in the proportion of eosinophils, basophils and lymphocytes, and in ECP levels in induced sputum collected from patients with occupational asthma (farmers, bakers) 22 h after allergen challenge as compared to baseline values.

Asthma is currently defined as a chronic inflammatory airway disease in which many cells, in particular mast cells, eosinophils and T lymphocytes play roles [1].

Several data seem to indicate a major role of eosinophils in occupational asthma induced by low- and high-molecular weight occupational agents. Obata et al. [9] described a significant change in sputum eosinophils during the late phase of allergic inflammation after plicatic-acid challenge in exposed subjects. Lemiere et al. [10] found a significant difference in sputum eosinophils and eosinophil-derived mediators in subjects with occupational airway allergy due to low-molecular weight agents. Walusiak et al. [11,12] observed a significant increase in the proportion of eosinophils and permeability index in the nasal lavage fluid after a specific challenge test with flours and baker's additives. High levels of ECP were found in sputum from patients with bronchial asthma [13,14].



It has been reported that the number of basophils is increased in the airways of asthmatic subjects as compared with normal persons and is also increased during asthma exacerbations [6,15]. These cells migrate to areas of inflammation in response to a specific stimulus. We found that the proportion of basophils was significantly increased in induced sputum during the late phase of allergic inflammation after the specific inhalatory challenge. These cells may be important for the development of the allergen-induced late asthmatic response [15].

Lymphocytes play a crucial role in the inflammatory processes of allergic asthma [1]. We noticed that the proportion of lymphocytes was significantly increased in induced sputum 24 h after the specific inhalatory challenge. Pizzichini et al. [16] found increased proportions of T lymphocytes in induced sputum obtained from mild asthmatics. There is an increasing evidence that lymphocytes play a major role in orchestrating the recruitment of airway eosinophils [17].

There was no significant increase in the percentage of albumin (mucosal/vascular permeability index) in induced sputum collected from subjects with occupational asthma after allergen provocation. Our observation is in agreement with data presented by Robinson et al. [18]. Hypertonic saline solution increases bronchovascular permeability, thus elevated albumin level in baseline induced sputum samples would obscure further allergen-induced increase.

Sputum ECP level was significantly higher after the provocation with occupational allergens compared to its value before the challenge. Some data indicate that either sputum eosinophils or eosinophil cationic protein increase during workshift or after the specific inhalatory challenge. This observation can be used as objective evidence to support the diagnosis of occupational airway allergy [10,18,19].

We have shown that histamine PC_{20} measured 24 h after allergen challenge strongly correlated with the ECP level and proportion of basophils in induced sputum obtained from occupational allergics before the specific provocation ($r = 0.51$, $p < 0.001$; and $r = 0.57$, $p < 0.001$, respectively). Our study demonstrates that bronchial responsiveness is a rather complex mechanism that is certainly sustained not

only by the recruitment of inflammatory cells, but also by the release of their mediators.

In contrast to Park et al. [20], we did not find significant neutrophilic infiltration after the provocation with flours and grains. These cells may contribute to grain dust-induced asthma. A recruitment of neutrophils to the lower respiratory tract is caused by endotoxin-induced chemotaxis and complement activation [21].

Contrary to subjects studied by Parks et al. [20], our subjects with diagnosed occupational asthma were exposed to both flours and grains, so that differences in morphological findings between our and their data could be partly related to different occupational exposure.

To sum up, sputum induction with analysis of cellular and mediator changes is a useful method for assessing airway inflammation in occupational asthma.

Eosinophils, basophils and lymphocytes are dominant cells in sputum of asthmatic patients sensitized to grains and flours after allergen inhalatory challenge.

In our opinion, the specific inhalation challenge with monitoring of morphological changes in induced sputum is a valuable method for the diagnosis of occupational asthma.

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