Possibilities of Early Diagnosis of Occupational Asthma

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Abstract: Occupational asthma is one of the most frequent occupational diseases of the respiratory tract in developed countries. Moreover, the diagnosis of occupational asthma is difficult because the confirmation of the occupational origin of the disease has an important impact on the career of the employee and many persons must involuntarily leave their work position. To avoid serious consequences, it is necessary to develop new methods which could disclose the incipient occupational asthma earlier than methods available nowadays or support the diagnosis in case of equivocal results (decrease in ventilatory parameters) of the bronchoprovocation tests.

Exhaled breath condensate (EBC) analysis is a new non-invasive method which appears useful in occupational asthma diagnostics. Leukotrienes as obstruction markers and 8-isoprostane as an oxidative stress marker could be analysed from EBC. The concentrations of leukotrienes and 8-isoprostane were described to be elevated in EBC of asthmatic persons. Monitoring of leukotrienes and 8-isoprostane concentration changes in the EBC during the bronchoprovocation tests with allergens could bring new information about the pathophysiological changes in airways during inhalation tests with allergens.

Induced sputum is a relatively non-invasive method which could be used in asthma diagnosis. The monitoring of the sputum cell count (especially changes of eosinophils) has a potential to be used for monitoring of asthma and during allergen challenge tests, too. The elevation of sputum eosinophils was described after allergen tests in several studies.

Introduction

In developed countries, occupational asthma is one of the most frequent occupational diseases of the respiratory tract. Its initiating agent is a substance (allergen) which is present at the workplace. Diagnosis of occupational asthma is difficult because the confirmation of the occupational origin of the disease has a negative impact on the other employee rating and many subjects must leave their present work position. That is why many patients dissimulate and overcome their symptoms. Consequently, they are examined by a physician when the disease is already fully developed.

The long-lasting studies, which followed the patients with occupational asthma after their elimination from the occupational allergen exposure, found out that the disease persists in most patients and the symptoms do not dissolve completely [1]. This could be caused by the late diagnosis of occupational asthma, advanced inflammation in the airways when the mucous membrane became more sensitive towards further allergens exposure. That is why the early diagnosis of occupational asthma is essential. Especially a follow-up of subjects with atopy and of those who are working with offensive allergens is of utmost importance. For this purpose, new methods which could disclose the incipient occupational asthma earlier or support the diagnosis in case of equivocal results of the bronchoprovocation tests, are crucial.
Diagnosis of occupational asthma

Occupational asthma is defined in the Supplement of the Government regulation Nr. 290/1995, i.e. the Czech List of Occupational Diseases, in the chapter III, item 10 as a disease, which develops at the workplace with the exposure to dust or gas particles with allergic or irritative effect. It is supposed that occupational asthma represents about 5–15% of newly diagnosed cases of bronchial asthma [2].

Diagnosis of occupational asthma is based on the verification and the confirmation of bronchial asthma, on the evidence of the relationship between the asthma onset and the work exposure, and on the proof of the sensitization of the patient to the allergen present at the workplace. The verification of occupational asthma can be performed by several ways.

1. The monitoring of the peak expiratory flow parameter (PEF) is the most frequently used way (because of the high safety) in the world. The diurnal variation of PEF higher than 20% during the workplace measurements in comparison with the periods of the elimination from the workplace is considered a positive finding. Another possibility is a monitoring, whether there is a change of the bronchial reactivity during the period of withdrawal from the workplace exposure comparing with the period of exposure at the workplace. In this case at least twofold concentration of inhaled agent to decrease FEV₁ by 20% (PC₂₀) in the period associated with work exposure as regards to the period of withdrawal is needed [3].

2. The specific bronchoprovocation tests with workplace allergens are considered to be the “gold standard” in occupational asthma diagnostics. The occupational allergen exposure can be performed either via direct inhalation of the commercially manufactured standardized allergen (for example flour allergen), or by exposure in the special exposure box with controlled air draw-off mechanism where the patient simulates his/her work at the workplace (for example varnishing). Another possibility is the exposure of the subject at his/her workplace, if it is not possible to find out or to bring the suspected occupational allergen. The positive reaction means the decrease in FEV₁ parameter more than 20% during 24 hours following the inhalation test with regard to the values before test [4].

Sometimes the condition of 20% decrease in FEV₁ parameter after the test is not realized but the value is near 20%. It could be mainly when the testing proceeds without withdrawal of corticosteroid treatment. In these cases some other objective methods could support the final resolution about the positivity of the test. There are two relatively new objective methods which could be helpful in that way- exhaled breath condensate analysis and the analysis of induced sputum.

Exhaled breath condensate

The beginning of exhaled breath condensate (EBC) analysis was in the 18th century, when Lavoisier explored the process of breathing via the exhaled breath analysis. The first works which concerned EBC in relationship to airways disease were
published the in the 80ties of the 20th century. They concentrated on the lung surfactant and products of lipid peroxidation. In the 90ties of the 20th century the spectrum of published articles was enlarged by IL-1b, IL-2, IL-6 and TNF. The studies focused on isoprostanes and prostaglandins in asthmatics afterwards appeared, followed by studies of patients with chronic obstructive pulmonary disease (COPD), cystic fibrosis and interstitial lung diseases [5].

The EBC is a rich source of substances (metabolites of arachidonic acid, proteins, hydrogen peroxide, nitro-reactants and many other substances), which reflect the airways environment and offer many possibilities for the diagnostics and monitoring of various respiratory diseases [5]. Mediators from the airways are released into the exhaled breath, which then condensates in the cooled sampling tube. The collection of the EBC is a simple, non-invasive method which can be performed in children and seriously ill patients and can be repeated several times over [6]. It is supposed that aerosol particles of the EBC reflect the composition of the bronchoalveolar fluid [5].

Collection of EBC samples
EBC samples could be collected using various devices. Generally, they are composed from the mouthpiece with a one-way valve connected to the sampling device, which is cooled either by ice or by liquid nitrogen. The commercially manufactured Eco-Screen (Jaeger, Hoechberg, Germany) is an electricity cooled system. The examined person breathes into the system through the mouthpiece, connected to a valve block, in which the inspiratory and expiratory air is separated. The air is cooled by the countercurrent system in a collecting part of the system, which is composed of the lamellar condenser and the sample collection vial from the inert material. The liquid condenses on the inner wall of the condenser and drops into the vial.

Contamination of the EBC samples
During the sampling, the EBC could be contaminated by substances from the environment or by endogenous substances from the mouth and upper airways, but the risk of endogenous contamination is small because the laminar circulation is not sufficient enough for the aerosol formation. The surface of the upper airways is smaller comparing to the lower airways, therefore the incidental contamination could be considered as not significant. On the contrary, some substances are present in high concentrations in saliva. Consequently, it is recommended to control the saliva contamination of the EBC by the measurement of α-amylase [5].

EBC analysis
Many analytic methods are used nowadays for the assessment of the substances in the EBC. It is possible to use the immunologic methods (EIA, ELISA, RIA) preferred for the financial reasons, the photometric methods (fluorescence,
chemiluminescence), the high-pressure liquid chromatography (HPLC), or the combination of mass spectrometry with liquid or gas chromatography (LC/MS, GC/MS). LC/MS or GC/MS appeared the most convenient for the leukotrienes and 8-isoprostane analysis because of their high specificity and ability to detect very low concentration of these markers (pg/ml). The disadvantage of these methods is a relatively high cost. Very sensitive ionization techniques are used too (negative chemical ionization), eventually monitoring of some specific ions in the spectrum of the measured substances (SIM – Selected Ion Monitoring). The decrease of the detection limit is possible because of the samples pre-treatment by lyophilisation or Solid Phase Extraction (SPE) [6].

Leukotrienes in EBC
Leukotrienes are metabolites of arachidonic acid. Arachidonic acid is 20-carbonic polyunsaturated fat acid that can be biotransformed both in enzymatic and non-enzymatic ways. The two main metabolic ways are dependent of the enzymes cyclooxygenase (COX) and 5-lipoxygenase (5-LO). Leukotrienes originate from the 5-lipoxygenase pathway. The initiation of the leukotrienes biosynthesis is dependent on the agonist stimulation (allergen associated IgE binding to the mast cell membrane), hydrolysis of arachidonic acid from the phospholipide membrane by phospholipase A$_2$ and the interaction of 5-LO with the 5-LO activating protein (FLAP). 5-LO catalyses the conversion of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (HPETE), which is converted to hydroxyeicosatetraenoic acid (HETE) or to the unstable epoxide – leukotriene A$_4$ (LTA$_4$). In alveolar macrophages, monocytes and neutrophils, LTA$_4$ is metabolized by hydrolysis to LTB$_4$. In mast cells, eosinophils and basophils (the main inflammatory allergic cells) the conversion of LTA$_4$ to leukotriene C$_4$ (LTC$_4$) takes place via LTC$_4$ synthase. LTC$_4$ is extracellularly metabolized by $\gamma$-glutamyltranspeptidase to leukotriene D$_4$ (LTD$_4$) which is biotransformed by dipeptidase to leukotriene E$_4$ (LTE$_4$). LTC$_4$, LTD$_4$ and LTE$_4$ are known as cysteinyl leukotrienes (cys-LT) or formerly slow reacting substance of anaphylaxis (SRSA). LTE$_4$ is the final metabolite of this leukotrienes line and is excreted in the urine. Its concentration in urine could be detected as the whole amount of the total body cys-LT production. The leukotrienes effect is connected with the G protein receptors BLT (for LTB$_4$) and receptors cysLT$_1$ and cysLT$_2$ (for cys-LT) [7]. CysLT$_1$ receptor is present on the pulmonary macrophages, smooth muscle cells, eosinophils, basophils, monocytes and mast cells, B lymphocytes and CD34 cell precursors; it was detected in the spleen, colon, placenta, pancreas, prostate, heart, brain, kidneys, liver and fat tissue. CysLT$_2$ receptor is present on the Purkyňe heart fibres, adrenal gland cells and brain cells, eosinophils and macrophages [8]. During in-vitro monitoring of the contractile capacity, approximately the same potential of LTC$_4$ and LTD$_4$ was described to initiate the smooth muscle airways constriction. The potential of LTE$_4$ was lower [9]. Cys-LT
could be considered as airways obstruction mediators [10]. CysLT$_2$ receptor is associated with the pulmonary vascular constriction in the lungs [7].

Cys-LT mediate bronchoconstriction, increases bronchial hyperreactivity, plasma exudation, they support Th2 cell response, suppress Th1 response, evoke eosinophils' infiltration and degranulation, hyperplasia of mucous cells, mucus hypersecretion, pulmonary basal corpuscles inhibition, smooth muscles hyperplasia, epithelial cells proliferation, they increase collagen deposition, and they exert vasodilatation or vasoconstriction.

The main activities of LTB$_4$ are chemotaxis of neutrophils, support of IgE synthesis and nuclear transcription.

Cys-LT production was in the past monitored only by the urine LTE$_4$ concentration. This concentration reflects the total body leukotrienes production, from which the airways production represents only a part. The EBC collection enables monitoring of the leukotrienes concentration changes namely from the airways and involves not only LTE$_4$ but also the main contractile leukotrienes LTC$_4$ and LTD$_4$ [9]. Based on the fact that cys-LT participate on the smooth muscle contraction, the concentration of LTE$_4$ as a main metabolite of cys-LT in urine was measured at rest and during non-specific and specific bronchoprovocation tests with occupational allergens. The concentration of LTE$_4$ in urine was monitored before and during bronchoprovocation tests with allergens and after aspirin administration in aspirin-sensitive asthmatics. After allergen exposure the elevation of LTE$_4$ in urine was found in atopic asthmatics [11]. Taylor [12] and Smith [13] described elevation of LTE$_4$ in urine during three hours after allergen inhalation. Manning [14] studied concentrations of LTE$_4$ during the early and late allergic reaction. During the early response the level of bronchoconstriction correlated with the elevation of LTE$_4$ concentration in urine; however during the late response the elevation of LTE$_4$ concentration was not significant. In contrast, O'Sullivan [15] described the elevation of LTE$_4$ concentration in urine both during the early and the late allergic reaction after the allergen inhalation. A significant elevation of LTE$_4$ concentration was described in aspirin sensitive asthmatics after aspirin administration in comparison to the level before the application [11, 16, 17].

Ono [18] described the elevation of cys-LT concentrations in the EBC after specific bronchoprovocation tests with allergen during the early allergic response together with increased concentration of LTE$_4$ in urine.

LTB$_4$ and cys-LT were detected in the EBC of healthy subjects, and comparing with them they were elevated in adult asthmatics with stable moderate to severe asthma [19]. In persons with aspirin sensitive asthma not treated by corticosteroids, the concentrations of cys-LT in the EBC were significantly higher than in aspirin-tolerating asthmatics not treated with corticosteroids. On the contrary, the cys-LT concentrations did not significantly differ between the groups of aspirin-tolerating asthmatics treated and not-treated by corticosteroids. Cys-LT
concentrations were higher in asthmatics than in healthy persons [20]. Mondini [21] found significantly higher LTE$_4$, LTB$_4$ and 8-isoprostane concentrations in asthmatic children with atopy (both treated and not-treated by corticosteroids) in contrast to the healthy persons. On the other hand, LTB$_4$ was found elevated in persons with COPD. Montuschi [22] described increased LTB$_4$ in the EBC in smoking persons with COPD both treated and not-treated by corticosteroids in comparison to healthy persons.

8-isoprostane in EBC

Isoprostanes originate from the non-enzymatic peroxidation of arachidonic acid [23]. F$_2$-isoprostanes are considered the best biomarkers of the oxidative stress and lipids' peroxidation in vivo as regards to their stability, specificity in endogenous peroxidation and relatively easy detection in biological samples [24]. Many their effects on the respiratory system are known, i.e. contraction of bronchial smooth muscles (in vitro) [25], obstruction of the airways with the plasma exudation in guinea-pigs in vivo [26]. In addition, isoprostanes as markers of the oxidative stress increase in subjects with interstitial lung diseases, what points to their possible utilization as potential mediators of the activity of certain pulmonary diseases caused by oxidative stress, such as pneumoconioses [27].

Increased 8-isoprostane concentration in the EBC was found in persons with stable asthma (in contrast to healthy persons). Moreover, its elevation correlated with the asthma severity and the degree of inflammation [28] in asthmatics with both aspirin-sensitive and aspirin-tolerating asthma, and also in asthmatic children both treated and not-treated by corticosteroids [29, 30]. There results support the fact that 8-isoprostane as a marker of oxidative stress is elevated in adults and children with asthma and this increase is relatively independent on the corticosteroid treatment [28].

8-isoprostane was increased in smokers and ex-smokers with stable COPD in comparison with healthy smokers and non-smokers [30]. It was described that during exacerbations of COPD 8-isoprostane concentration increased and subsequently decreased after the antibiotic treatment [31]. No correlation was found between ventilatory parameters and 8-isoprostane in the EBC either in asthmatics or COPD patients [30], but negative correlation was found for FEV$_1$ in patients with cystic fibrosis [32].

Utilization of EBC analysis in occupational asthma diagnostics

In several above mentioned studies, LTE$_4$ in urine and cys-LT in EBC were described elevated after bronchoprovocation tests with allergens. Cys-LT are considered as markers of airways obstruction so their elevation could support the positive result of the bronchoprovocation test in case of borderline decrease of ventilatory parameters (not more than 20% decrease of FEV$_1$ after the test). This could be crucial in the occupational asthma assessment.
In our department a pilot study with 47 persons who underwent bronchoprovocation tests with occupational allergens was performed. In 3 patients with the late asthmatic reaction LTC4 was elevated before and/or at the time of the maximum decrease in FEV1. In 3 patients with an early asthmatic reaction no important changes were observed [33]. LTC4 could be a helpful marker in occupational asthma diagnostics in our conditions but there is a need of more trials before any conclusion is done because the tested group was very small.

**Induced sputum**

Sputum induction (sputum obtained using inhalation of saline solutions) is not a completely new method in medicine. This method was used in the 50ies of the 20th century by Bickerman for obtaining sputum which was used in the lung cancer diagnostics. In the 90ies of the 20th century the method of the induced sputum was modified by Pin and Fahy and the epoch of using of the induced sputum in diagnostics and treatment of bronchial asthma started [34]. The induced sputum analysis can bring the data both about cells and inflammatory markers (analysis of sputum supernatant). The information about the inflammation in the lower respiratory tract can be obtained also from the bronchoalveolar lavage fluid, bronchial wash or bronchial biopsy. However, these examinations are invasive and less tolerated by the patients. In the comparison of these methods a good correlation was found between eosinophils in induced sputum and in bronchoalveolar lavage fluid, unlike with eosinophils from the bronchial biopsy [35].

The idea of sputum induction results from the fact that not all persons are able to produce sputum spontaneously. That is why sputum was induced using the inhalation of the hypertonic saline solutions (sodium chloride, NaCl). The most frequently recommended concentration is 4.5%, because it is produced commercially [36]. The inhaled saline concentration can be subsequently increased, too, if it is well tolerated by the patient (the most frequent combination is 3%, 4%, and 5% NaCl). This schema is feasible for patients with increased bronchial hyperresponsiveness where increased caution is required. In patients with higher risk of bronchospasm it is possible to start the inhalation with the isotonic physiological saline solution (0.9% NaCl). The highest described NaCl concentration used for sputum induction was 7% [37]. The hypertonic saline is more effective in sputum induction than the isotonic saline; however the cell distribution in sputum is not significantly different [38]. Another substance which was used for sputum induction was uridine-5’-phosphate. A lower decrease of ventilatory parameters and a greater amount of sputum were described after using this substance in comparison with NaCl [39].

Ultrasound nebulisers are mainly recommended for sputum induction because most of other nebulisers do not have an adequate output. The required output of the nebuliser is approximately 1 ml/min [36].
During sputum induction it is necessary to monitor ventilatory parameters (the parameter FEV$_1$ is usually monitored during sputum induction.). In persons with the increased bronchial reactivity the hypertonic saline solution could cause a decrease in ventilatory parameters and bronchoconstriction. These undesirable effects could be prevented by the application of salbutamol (200–400 µg) or other $\beta_2$-agonist before saline inhalation. Induced sputum has already been analysed in many trials and the effect of pre-treatment by $\beta_2$-agonists on the sputum cell count has been studied. It was not confirmed that the pre-treatment by salbutamol significantly influenced the sputum cell count [38, 40]. The effect of salbutamol pre-treatment on sputum ECP concentration was not found either, contrary to histamine concentrations which decreased after this pre-treatment [40].

The induced sputum could be processed as a whole sample (with saliva) or as selected sputum (selected plaques of sputum). Both methods have their advantages and disadvantages. The non-selected sputum method is relatively faster but the sample could be more contaminated by the epithelium cells from mouth. The selected sputum method is more difficult for the laboratory processing but the samples are better for the visual microscope cell readout.

The induced sputum cell count contributes to the diagnosis of respiratory diseases. The total sputum cell count increases in the uncontrolled asthma, in smokers and due to bacterial infections [41, 42]. Bronchial asthma or eosinophilic bronchitis and allergen exposure in allergen sensitive persons increase the number of eosinophils in sputum. Up to 80% of asthmatics not-treated by corticosteroids and up to 50% of steroids treated asthmatics with asthmatic symptoms have increased eosinophils in sputum [43, 44, 45]. On the other hand, in chronic bronchitis, COPD, in smokers, in respiratory infections, in ozone and other air pollutants exposure, in endotoxins exposure and in non-eosinophilic steroid-resistant bronchial asthma, the neutrophils become elevated in sputum. Increased sputum lymphocytes were described in sarcoidosis and pneumonia caused by Chlamydia [46].

Although induced sputum method was originally developed for the detection of the infectious agents, in last years this method appeared to be very useful for the study of asthma and COPD. The study of Pin demonstrated increased number of eosinophils and metachromatic cells in sputum count of asthmatics contrary to the healthy subjects [47]. Fahy found that the acellular part of sputum from asthmatics contained an increased concentration of ECP and albumin in comparison to healthy subjects [48, 49]. The increased number of eosinophils in sputum before the start of treatment is also a good predictor of fruitfulness of corticosteroids treatment [50]. Chlumský [51] described a correlation between the level of the bronchial obstruction and percentage of eosinophils in sputum. The presence of eosinophils in sputum in patients treated with inhaled corticosteroids pointed on the chronic persisting inflammation which deficiently answered to the inhaled corticosteroids treatment and frequently required the treatment with systemic corticosteroids [51].
Utilization in occupational asthma diagnosis

Several studies describing utilization of induced sputum in occupational asthma diagnostics were published. Lemiere [52] studied changes of cell parameters in induced sputum during specific bronchoprovocation tests and described a significant increase of the eosinophils and neutrophils, and a decrease of macrophages percentage in sputum 7 hours after the exposure to occupational allergens (high molecular weight (HMW) and low molecular weight (LMW) allergens) in patients who positively answered to the specific bronchoprovocation test. In subjects with a negative bronchoprovocation test a significant increase of eosinophils in 2 from 14 persons was also found, but this increase was not as high as after a positive test. Lemiere [53] examined also the changes in sputum after gradually increasing allergen concentrations in patients with previously diagnosed occupational asthma concurrently with the ventilatory parameters monitoring. The increase of sputum eosinophils preceded a significant decrease of ventilatory parameters (bronchoconstriction) and was more pronounced in patients exposed to LMW allergens. Obata [54] described a significant increase of the sputum eosinophil percentage 6 and 24 hours after the positive bronchoprovocation test in 9 persons exposed to the red cedar (plicatic acid as LMW allergen). After a negative challenge test a significant increase of sputum eosinophils was found in 3 from 8 persons. Changes in induced sputum were also monitored before and during the exposure to suspected allergens at the workplace (4 weeks of exposure) and after elimination from the workplace allergens exposure (4 weeks outside the workplace). Sputum induction was performed up to 48 hours after the last workplace exposure and after 4 weeks outside the workplace. The patients with occupational asthma had significantly increased sputum eosinophils and sputum ECP during the workplace exposure contrary to the period of their elimination from the workplace [55].

In our department the non-selected and selected method of sputum processing was tested. If the method of non-selected sputum was used, we did not found the elevation of sputum eosinophils neither after the positive specific test with the occupational allergen or after negative tests [56]. Moreover many samples of sputum were highly contaminated by the epithelium cells from mouth. In comparison the selected sputum method was more effective. In the pilot study with 23 persons we found the significant increase of sputum eosinophils in the group of 7 persons with positive result of the bronchoprovocation test with the occupational allergen. Moreover in two persons with the negative result of the test (the decrease in FEV₁ did not reach 20%) the significant elevation of eosinophils was found too [57].

The monitoring of induced sputum shows that in most of the sensitised people the sputum eosinophils increase due to the exposure to the occupational allergen. In the literature this elevation is mainly described for LMW allergens. It is not
evident why some allergens cause sputum eosinophils elevation and some not despite the evident bronchoconstriction after the allergen application.

The elevation of sputum eosinophils supports the positive results of the allergen bronchoprovocation test. In persons with the negative bronchoprovocation test (not significant decrease of FEV$_1$), the elevation of sputum eosinophils after the test supports the idea of the continuing in the allergen exposure – the positive result of the test (significant decrease of FEV$_1$) could be reached after the repeated exposure to the occupational allergen.

**Conclusion**

Two promising methods which could bring new information to asthma (not only occupational) diagnostics were described in this review article. The contribution of these methods to the investigation of asthma pathophysiology was confirmed by many studies. Obviously, these methods could also be used in occupational asthma diagnostics which is particularly difficult. The effect of exposure to specific allergens both on the sputum cell count and on the concentration of leukotrienes and 8-isoprostanate in the EBC could contribute to a better knowledge of the pathophysiology of occupational asthma.

**References**


Diagnosis of Occupational Asthma


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