

Original article

Analysis of exhaled leukotrienes in nonasthmatic adult patients with seasonal allergic rhinitis

Background: Leukotrienes (LTs) are increased in exhaled breath condensate (EBC) in patients with asthma. So far no data have been reported about LT levels in nonasthmatic patients with seasonal allergic rhinitis (SAR). The aim of the study was to find out whether the LT levels in EBC were increased in the nonasthmatic adult patients with SAR both during and after the pollen season in comparison with healthy controls and to assess the changes of the LT levels after the pollen season.

Methods: Twenty-nine nonasthmatic adult patients with SAR underwent measurement of exhaled LTs in the EBC during and after the pollen season. Leukotrienes B₄, C₄, D₄ and E₄ were analysed by a specific and sensitive gas chromatography/mass spectrometry (GC/MS) assay and compared with 50 healthy nonsmoking controls. Spirometry, skin prick tests and nonspecific IgE were evaluated.

Results: Leukotrienes concentrations (B₄, E₄ but not D₄) were significantly increased in and after the pollen season in patients with SAR in comparison with healthy controls. In most of the samples, LT C₄ was undetectable. The values of all exhaled LTs were significantly decreased after the pollen season compared with the seasonal baseline: LTB₄ ($P = 0.023$), LTD₄ ($P = 0.020$), LTE₄ ($P = 0.047$).

Conclusions: Levels of exhaled LTB₄ and LTE₄ were higher in SAR patients than in healthy controls and decreased after the pollen season as compared with levels in season. The SAR patients with the highest in season LT levels had also the post-season levels elevated and this may be an early marker of inflammatory process in the lower airways despite the absence of clinical symptoms of asthma.

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There are sufficient data linking allergic rhinitis and asthma to such an extent that allergic rhinitis is taken as a serious risk factor for asthma (1, 2). The chronology of rhinitis and asthma is still under discussion. Some studies have examined the bronchial mucosa in nonasthmatic patients with allergic rhinitis, pointing out that there was slightly increased in the basement membrane size and a moderate eosinophilic inflammation (3–5). The inflammation in asthma presents a complicated and diverse process and there is no single mediator which could be used as an universal marker of disease activity and progression. On the other hand, the leukotrienes (LTs) belong to well-known mediators of inflammation. LTC₄, LTD₄, and LTE₄ (so-called cysteinyl LTs) are potent pro-inflammatory mediators in asthma. LTB₄ has weak effects on smooth muscle, but it may contribute to airway narrowing, increasing mucus secretion and producing local edema (6). LT modifiers are currently used in the therapy of asthma.

Leukotrienes have been measured in plasma (7), urine (8, 9), induced sputum (10), bronchoalveolar lavage (BAL) fluid (11, 12) and exhaled breath condensate (EBC) in patients suffering from asthma. In comparison with the previous methods, EBC is a method to sample secretions from the airways which is absolutely non-invasive and potentially useful for quantifying lung inflammation in patients with pulmonary diseases (13). CysLTs and LTB₄ are increased in EBC in both adults and children with asthma compared with healthy controls (14–16).

In our study, a specific and sensitive gas-chromatography/mass spectrometry (GC/MS) was used to measure the cysteinyl LTs and LTB₄ concentrations in EBC collected from nonasthmatic adult patients with seasonal allergic rhinitis (SAR) both during and after the pollen season as well as from healthy control subjects. The aim of the study was to find out whether there were SAR nonasthmatic patients with increased levels of exhaled

LTs in comparison with the healthy controls and to assess changes of the LT levels after the pollen season.

In accordance with ARIA recommendations, the patients with persistent rhinitis should be evaluated for asthma. Examination of exhaled LTs could enable an early detection of an inflammatory process in the lower airways in patients with SAR without clinical symptoms of asthma.

Methods

Study subjects

Two groups of subjects were investigated: 29 (11 male, 18 female) adult outpatients with SAR with no symptoms of asthma and normal spirometry and 50 (14 male, 36 female) healthy nonatopic adults. All subjects were nonsmokers. Demographic characteristics are summarized in Table 1. The controls were nonatopic healthy subjects recruited from the hospital staff without history of allergic rhinitis, asthma or other chronic airway diseases, without respiratory infection in the previous 4 weeks. The study subjects were sequentially enrolled among the outpatient clinic patients treated for SAR symptoms during the grass pollen season. They were selected according to the prespecified inclusion and exclusion criteria. All subjects of the study group with allergic rhinitis belonged to the persistent and moderate/severe category as defined in the new ARIA guidelines (1). This study was approved by the Ethical Committee of the Na Homolce Hospital in Prague. EBC samples of the patients were collected and measured twice, for the first time during the pollen season (June) and secondly after the pollen season (November). The diagnosis and classification of SAR was based on the correlation between clinical history, physical examination and skin prick tests (SPT). Nonspecific IgE levels in the serum of patients suitable for the study were measured (Chemiluminiscence; Immulite DPC, Bad Nauheim, Germany), afterwards the patients were asked to record the SAR symptom score on a five-category scale (0, no symptoms; 1, mild; 2, moderate; 3, severe; 4, very severe). Allergic sensitization was determined by SPT reaction to allergens on the volar surface of the forearm. The allergens were grass mixture, betulaceae (birch, alder, hazel) *artemisia vulgaris*, ambrosia, *cladosporium*, *aspergillus* mix, *alternaria*, *dermatophagoides pteronyssinus* and *farinae* (Stallergen, Antony, France). The negative control was glycerol-buffer diluent and positive control histamine (1 mg/1 ml). The reaction was recorded (15-min after administration) and judged positive when the wheal diameter was 3-mm larger than the negative control. All patients were shown to be highly positive to grass pollen, none of them was sensitive to the *dermatophagoides* or moulds and 17 of them were sensitive to birch,

but without symptoms during the birch pollen season. None of the patients was positive to the other tested pollens (*artemisia*, and *ambrosia*).

The inclusion criteria were as follows: at least 2 years of clinical history of moderate to severe SAR. All patients had to have a positive SPT to grasses and suffered from symptoms of allergic rhinitis only during the grass pollen season. Normal pulmonary function parameters (spirometric parameters: FEV₁, FVC, MEF_{25–75}) and no symptoms of asthma were required. SPT with positive reaction to birch was allowed, but no symptoms in time of birch pollen. Exclusion criteria were: bronchial asthma, sensitization to other seasonal or perennial aeroallergens (positive SPT to moulds, mites or animals, *ambrosia* and *artemisia*), chronic bronchitis, smoking history, respiratory tract infections in the previous 4 weeks, the use of leukotriene receptor antagonists in the previous 4 weeks or nonsteroidal anti-inflammatory drugs in the previous 2 weeks or any nasal steroids in the previous month. Only the use of antihistamines was allowed.

Healthy controls were not taking any medications and none of them were smokers. In all subjects there was neither history of significant airway disease and atopy nor respiratory infection in the previous 4 weeks. These healthy individuals had to pass spirometry examination with normal outcomes.

Pulmonary function

All measurements were carried out using the spirometer Master-screen (Jaeger, Hochberg, Germany) and the best value of three manoeuvres was expressed as an absolute value and as a percentage of the predicted value.

Exhaled breath condensate collection

Exhaled breath condensate samples were collected using a condenser EcoScreen (Jaeger) (13) into a special plastic tube. Subjects breathed tidally through a mouthpiece connected to the condenser for 15 min while wearing a noseclip. The volume of the EBC samples ranged from 1 to 2 ml. Samples of EBC were stored at -70°C for no longer than 2 months before LT measurements and thawed only once before the analysis.

Amylase assay

To check for saliva contamination of EBC, amylase activity in EBC samples was assayed by an α -Amylase-Liquid BIO-LA-TEST kit (Pliva-Lachema, Brno, Czech Republic). The maximum assayed amylase activity in EBC was less than 1% of its activity in the saliva providing the evidence of no contamination of EBC samples with saliva.

Assay for leukotrienes in EBC

LTB₄, LTC₄, LTD₄ and LTE₄ concentrations in EBC were measured using gas chromatography-mass spectrometry after derivatization with ethylchloroformate as described elsewhere (17). The derivatization method was previously successfully used for a gas chromatographic analysis of amino acids, hydroxycarboxylic acids, fatty acids and dicarboxylic acids (18). Arachidonic acid was used as an internal standard. Separation was performed on a fused-silica capillary column 8 m $\times$ 0.25 mm of Optima δ -3 type (Macherey-Nagel GmbH & Co., Düren, Germany) in the split-less mode. Tandem GC/MS (Finnigan Mat Co., San Jose, CA, USA) was used in selected ion monitoring mode for LT identification and quanti-

Table 1. Demographic characteristics of the study subjects

	Healthy subjects	SAR patients
<i>n</i>	50	29
Age (years)	37.6 $\pm$ 11.0	29.2 $\pm$ 8.8
Age (range)	16–55	19–60
Disease duration (years)	–	14.2 $\pm$ 9.9
Disease duration (range)	–	2–35
Sex (M/F)	14/36	11/18
FEV ₁ (% predicted)	107.3 $\pm$ 12.7	95.4 $\pm$ 14.6
FVC (% predicted)	104.5 $\pm$ 13.5	93.7 $\pm$ 15.2
MEF _{25–75} (% predicted)	100.7 $\pm$ 20.2	91.9 $\pm$ 20.5

Data are shown as mean $\pm$ SD.

fication. The detection limit at the signal to noise ratio of 5 was 1 pg/ml. The linearity of the detector response was confirmed over the concentration range of 5.0–1000 pg/ml. The recovery of all analytes from EBC samples was higher than 90%. The coefficients of variation of repeated LT measurements in spiked quality control samples or in EBC samples which were analyzed in duplicates were less than 10%. Total IgE was measured by chemiluminescence using a monoclonal immunometric assay (Immulite DPC).

Statistical analysis

The data were statistically analysed by nonparametric tests. Medians and interquartile ranges were used as measures of location and variability of LT concentrations. Wilcoxon's ranks test was performed for testing the hypothesis of no difference between the medians of LT concentrations during and after the pollen season. Mann–Whitney test was used to assess whether two populations have the same medians. Kruskal–Wallis one-way analysis of variance was used for testing the hypothesis that there is no difference between subjects in three or more groups. Correlations between variables were evaluated by Spearman's correlation coefficient (r). Significance was defined as a value of $P < 0.05$.

Results

The GC-MS method was able to detect LTB_4 , LTD_4 , and LTE_4 in EBC samples from both patients with SAR and healthy controls. The quantitatively prevailing LTs were LTB_4 and LTE_4 . Levels of LTD_4 were approximately 10- to 15-times less than those of either LTB_4 or LTE_4 . Concentrations of exhaled LTC_4 in most samples were less than the limit of detection of 1 pg/ml.

Median concentrations of LTB_4 and LTE_4 were higher in patients with SAR both during and after the pollen season [LTB_4 : during the season 97 (interquartile range 61–203) pg/ml, $P < 0.001$, after the season 93 (68–184) pg/ml, $P < 0.001$; LTE_4 : during the season 87 (61–197) pg/ml, $P = 0.036$, after the season 82 (55–166) pg/ml, $P = 0.028$] as compared with those of healthy subjects [LTB_4 : 79 (62–112) pg/ml; LTE_4 : 74 (35–112) pg/ml] (Fig. 1). There were no significant differences in the median levels of LTD_4 between the controls [8 (2–16) pg/ml] and the patients with SAR measured either during the pollen season: [8 (2–24) pg/ml; $P = 0.79$] or after the season [6 (2–15) pg/ml; $P = 0.29$] (Fig. 1). After the pollen season, statistically significant decreases were found in the median levels of all LTs in the EBC samples from SAR patients (LTB_4 : $P = 0.023$, LTD_4 : $P = 0.020$, LTE_4 : $P = 0.047$). However, the median postseasonal levels of LTB_4 and LTE_4 in SAR patients remained higher than those from healthy subjects. In 10 SAR patients with highest in-season levels of exhaled LTB_4 and LTE_4 , the post-season values remained higher than the maximum levels observed in healthy subjects (Fig. 1).

In patients with SAR there was a strong and statistically significant ($P < 0.001$) correlation during the pollen season between LTB_4 concentrations and cysteinyl LTs

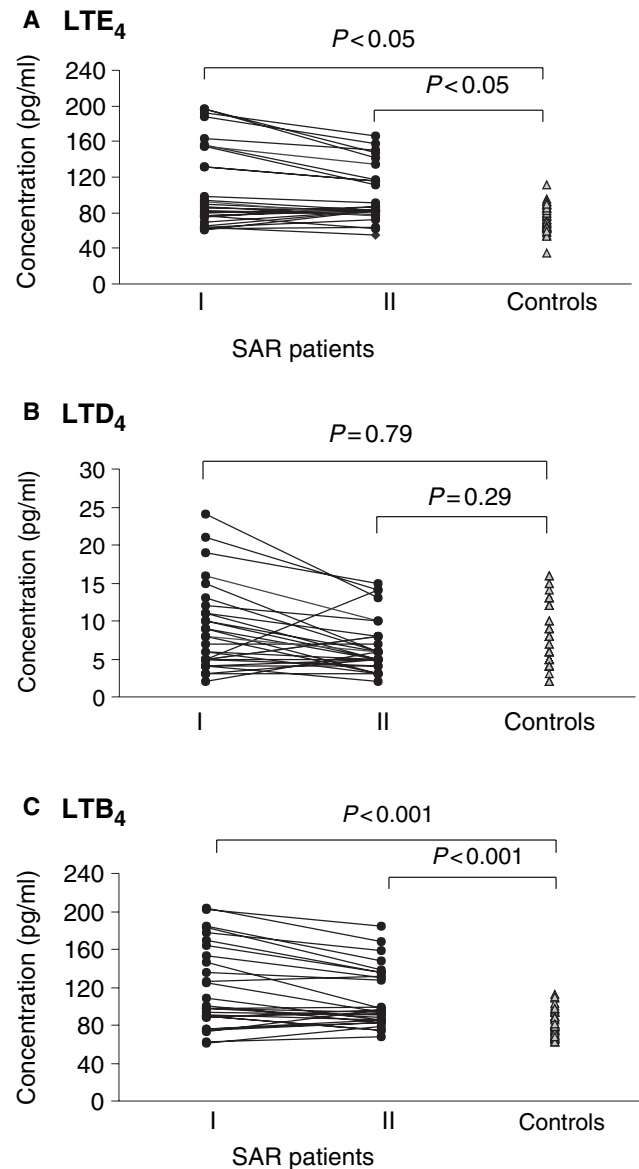


Figure 1. Concentrations of leukotrienes in the exhaled breath condensate of patients with seasonal allergic rhinitis (I, during the pollen season; II, after the pollen season) and of healthy subjects.

and moderate correlation between cysteinyl LTs (LTD_4 and LTE_4) mutually ($P < 0.01$) (Table 2). Correlations after the season were somewhat weaker, especially between LTB_4 and LTD_4 (Table 2). Healthy controls showed fairly weak correlation between LTB_4 and LTE_4 . The two other correlations were almost negligible (Table 2).

Correlations between LTs in SAR patients during and after the grass pollen season were substantial (LTB_4 : $r = 0.85$, LTD_4 : $r = 0.53$, LTE_4 : $r = 0.77$) and statistically significant ($P < 0.01$). No considerable correlations were found between any of the LTs levels, total IgE or

Table 2. Spearman's correlation coefficients and the corresponding *P*-values of the relationships between the concentrations of leukotrienes in patients with seasonal allergic rhinitis and healthy subjects

	Patients with seasonal allergic rhinitis		Healthy controls
	During the season	After the season	
LTB ₄ and LTD ₄	0.73 (<i>P</i> < 0.001)	0.42 (<i>P</i> = 0.022)	−0.04 (<i>P</i> = 0.773)
LTB ₄ and LTE ₄	0.89 (<i>P</i> < 0.001)	0.79 (<i>P</i> < 0.001)	0.35 (<i>P</i> = 0.012)
LTD ₄ and LTE ₄	0.74 (<i>P</i> < 0.001)	0.56 (<i>P</i> = 0.002)	−0.08 (<i>P</i> = 0.585)

any of the lung function parameters (i.e. FEV₁, FVC, MEF_{25–75}) during the pollen season.

The LT levels in patients were associated with the SPT reactivity. Based on the results of SPT reactivity the subjects were divided into four categories. None of them had the lowest reactivity. Median concentrations of LTs in the patients with the low SPT reactivity were seemingly lower in comparison with those in the patients with the moderate and higher SPT reactivity. However, the differences were not statistically significant (Kruskal–Wallis test).

Discussion

In this study, we have measured concentrations of the leukotrienes LTB₄, LTC₄, LTD₄ and LTE₄ in EBC samples obtained from a group of healthy individuals as well as from the patients who visited our outpatient clinic due to SAR symptoms. The patients were treated with second-generation antihistamines only. In patients with SAR, second measurements were performed after the pollen season when they experienced no symptoms of SAR. We have found that both in- and postseason levels of LTB₄ and LTE₄ were higher in patients with SAR compared with those of healthy nonatopic subjects while LTD₄ levels did not differ significantly. It appears that LTD₄ is not a sufficiently sensitive marker in relation to allergen exposure. Among the family of LTs, our interest therefore was focused on LTE₄ as the end product of the cysteinyl LT cascade and LTB₄. Ten SAR subjects had in-season levels of both LTB₄ and LTE₄ increased by 25–100% above maximum concentrations observed in healthy subjects. In those subjects, the LT concentrations remained elevated also after the pollen season.

These findings correspond with the measurements of exhaled NO, another marker of lower airway inflammation in patients with SAR. Henriksen *et al.* (19) have found elevated NO levels in subjects with allergic rhinitis in the nonpollen season, especially in perennially sensitized subjects, as compared with healthy controls. Exhaled NO was increased in the pollen season mainly due to a twofold increase in those with seasonal sensitization. Nasal NO was not significantly different from controls out of pollen season and did not increase significantly in the pollen season. The authors concluded

that an increase in exhaled NO on allergen exposure, particularly in hyperresponsive subjects with allergic rhinitis, may be suggestive of airway inflammation and an increased risk for the development of asthma.

The anatomic and cellular site of origin of various biomolecules in the EBC is still under investigation. Large nonvolatile and relatively lipophilic molecules such as leukotrienes are added to the exhaled breath mainly in small aerosol particles which are further diluted with exhaled water vapours. As subjects in our study wore a nose clip during the collection procedure, the nasal contribution to the EBC contents was prevented and the levels of leukotrienes most probably reflect increased production in the lower airways. Cys-LTs are mainly produced by mast cells and macrophages constitutively present in the airways. Eosinophils, which are not present in the airways of healthy subjects, migrate into the airways of asthmatic patients and may synthesize cys-LTs, as well as LTB₄ (1). Neutrophils are the cellular source of LTB₄. The apparent correlation between LTD₄ and LTE₄ both during and after the season is consistent with a common metabolic pathway of these LTs. A strong correlation between LTB₄ and cysteinyl LTs is more surprising because these LTs reflect the involvement of different mechanisms in the airway inflammatory process. We observed similar correlation in patients with asthma treated with inhaled steroids (17).

Several *in vitro*, as well as clinical studies have documented increased production of cys-LT and LTB₄ by peripheral blood cells, dispersed cells from human nasal polyps or in nasal washes of subjects with allergic rhinitis (20, 21). In some of the studies, antihistamines exerted inhibitory effects towards leukotriene synthesis (22–24). In our study, we observed an increased concentration of LT in EBC of patients with symptoms of SAR despite the treatment with second-generation antihistamines. Thus, our patients with insufficient SAR control with antihistamines might potentially benefit from addition of LT modifiers. Results from recent studies of leukotriene antagonists, including combination therapy of antihistamines and LT antagonists, demonstrated improved nasal rhinorrhea, sneezing, and congestion, providing a growing rationale for the use of LT receptor antagonists to treat allergic rhinitis (25–27).

Increased levels of inflammatory markers in EBC may reflect underlying inflammation of the lower airways. This hypothesis requires further verifications and studies. There was no relationship between the LT levels and lung-function parameters. We attempted to correlate the LT levels in EBC with the following characteristics of patients: history of SAR, symptoms score, skin test scores (weal and flare size) or degree of reactivity to grasses and scale of sensitization (monosensitization or polysensitization respectively SPT positivity to grasses only or plus mixture: birch, hazel and alder) and the level of nonspecific IgE. The patients referred their symptoms scores between the third and fourth degree on the 5-item scale

and, could therefore not be stratified based on this characteristic.

Among all characteristics, only the degree of reactivity in SPT showed the suggestion of an association with LT concentrations. We expected that the intensity of pollen sensitization in patients with SAR could be connected to production of LTs in the lower airways. However it was not confirmed definitely. No correlation was proven between LT concentration and history of SAR, symptom score, nonspecific IgE or scale of sensitization. In further studies, the SAR patients with elevated leukotriene levels should undergo testing of bronchial hyperresponsiveness (BHR) in order to disclose underlying asthma.

In conclusion, it was found that levels of exhaled LTB₄ and LTE₄ were higher in SAR patients than in healthy

controls and decreased after the pollen season as compared to levels in season. The SAR patients with the highest in-season LT concentrations (25–100% above the maximum level in controls) consistently showed elevated levels also after the pollen season. This may be an early marker of inflammatory process in the lower airways despite the absence of clinical symptoms of asthma. It could be worthwhile to compare the LT levels in those patients with results of measurements of BHR or other indicators of inflammation such as exhaled NO.

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