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SHORT SURVEY

RANTES in exhaled breath condensate of stable and unstable asthma patients

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Summary
RANTES has been implicated in the allergic inflammation of asthma by promoting the migration and activation of the inflammatory cells, including eosinophils. The study was undertaken to evaluate RANTES levels in the exhaled breath condensate (EBC) of asthmatics with different degrees of asthma severity.

EBC was collected from 33 patients with allergic asthma (11 with steroid-naïve mild asthma, 10 with ICS-treated, stable mild-to-moderate asthma, 12 with ICS-treated unstable, severe asthma) and seven healthy volunteers.

In the three groups of asthmatics, RANTES concentrations in EBC were significantly higher compared with healthy volunteers. RANTES levels were significantly higher in patients with unstable asthma than in the two groups with stable disease. We observed statistically significant correlations between the concentrations of RANTES in EBC and F_{ENO} in the three studied groups of asthmatics; notably, the correlation between the parameters described above was strong positive in the group of unstable and steroid-naïve stable asthmatics. We also discovered a significantly positive correlation between RANTES in EBC and the serum ECP or blood eosinophil count in the groups of asthmatics with severe, unstable asthma and between RANTES and serum ECP in the group of steroid-naïve stable asthmatics.

Measurements of RANTES in EBC may provide another useful diagnostic tool for detecting and monitoring inflammation in patients with asthma.

Introduction

Chemokines are a family of cytokines that are believed to be involved in the pathogenesis of asthma, possibly by recruiting leukocytes to the inflammatory site.[1] The CC chemokines, such as RANTES and eotaxin, have been implicated in the allergic inflammation of asthma by promoting

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the migration and activation of inflammatory cells, including eosinophils.[2]

In many studies performed using invasive (such as bronchoalveolar lavage — BAL) as well as semi-invasive (e.g. induced sputum) methods in asthmatic patients, an increased expression of local and systemic CC chemokines has been demonstrated.[3,4] However, these relatively invasive approaches are unsuitable for repeated monitoring of airway inflammation.

By contrast, exhaled breath condensate (EBC), collected by cooling exhaled air, is a non-invasive, easily performed, rapid and effort-independent method for obtaining samples from the lower respiratory tract. There has been increasing interest in measuring EBC — a very useful method, especially in the assessment of inflammatory mediators related to the bronchial epithelium, in the pathophysiology and evaluation of new strategies for the treatment of asthma.[5]

The aim of the study was to assess RANTES concentrations in the EBC of asthmatics with different degrees of asthma severity, and to establish the possible correlation of these measurements with the other parameters of airway inflammation.

Material and methods

Patients

The study was performed upon groups of 11 steroid-naïve mild allergic asthma patients; 10 patients treated with inhaled corticosteroids (ICS) with stable, mild-to-moderate allergic asthma; and 12 ICS-treated patients with severe, unstable allergic asthma. Asthma was diagnosed according to the criteria recommended by the GINA 2002.[6] The steroid-naïve asthmatics have not been treated with ICS. They were free from acute exacerbations and respiratory tract infections during the 3 months prior to the study. The patients with stable, mild-to-moderate asthma had been treated with low to medium doses of ICS at a constant dose for at least 3 months. Stable asthma was defined as a minimal need for rescue medications (short-acting β_2-agonists), no exacerbations, and no use of systemic steroids in the previous 12 months. The patients with severe, unstable asthma had required one or more hospitalizations for asthma and more than three oral steroid bursts in the last year. They had been taking high-doses of ICS and long-acting β_2-agonists for at least 6 months. Patients who had respiratory tract infections in the last month before the study were excluded from this study. All the patients were atopic and sensitized to common inhaled allergens, as evaluated by skin prick tests.

Seven healthy subjects were recruited for the study as a negative control group. They were free of respiratory tract infection within 3 months prior to the study and from other significant illnesses known to affect exhaled nitric oxide (F_{ENO}) measurements. Asthma patients and healthy volunteers were non-smokers and during the last year had not been passive smokers.

All of the patients and healthy volunteers were examined by a physician, then underwent EBC collection, F_{ENO} measurement, and spirometry. Blood samples were collected to determine serum total IgE, ECP and blood eosinophil count.

The study protocol was approved by the Ethics of Research Committee of the Medical University of Bialystok, number of agreement: R-I-003/80/2006. Informed consent was obtained from every patient entered in the study.

Measurements

Exhaled nitric oxide (F_{ENO}) was measured by the chemiluminescence technique using a Sievers 280i NO Analyzer (Boulder, Colorado, USA). The measurements were performed at an expiratory flow of 50 ml/s according to ATS recommendations for on-line measurement of F_{ENO} in adults.[7]

The spirometry (FEV_1) was performed using a MasterScreen Pneumo PC spirometer (Jaeger, Hoechberg, Germany), according to ATS standards.[8]

EBC was collected by using a commercially available condenser (EcoScreen; Erich Jaeger GmbH, Hoechberg, Germany) according to the current ATS/ERS guidelines.[5]

All measurements were performed at the same time (between 8.00—10.00 am) to avoid possible circadian rhythm of mediator concentrations in EBC. All patients were asked to refrain from eating and drinking before EBC collecting.

Exhaled air entered and left the chamber through one-way valves and the inlet and outlet, thus keeping the chamber closed. A low temperature inside the condensing chamber throughout the collection time produced a cooling down sample. The temperature of collection was around 0 °C.[5,9] Patients were instructed to breathe tidally for 10 min with nose clip. The respiratory rate ranged from 15—20 breaths/minute. Patients were asked to swallow their saliva periodically and to temporarily discontinue collection if they needed to cough. At the end of collection 1.5 to 3.5 ml aliquots of condensate were transferred to Eppendorf tubes and immediately frozen. Samples were stored at −80 °C.[10]

Serum total IgE concentrations and serum ECP were measured using ImmunoCAP™ Technology (Pharmacia Diagnostics, Uppsala, Sweden). The minimum detectable level of ECP was 2.0 μg/l. Blood eosinophil count was measured using a hematologic analyzer (Coulter Electronics GmbH, Miami, Florida, USA). The concentrations of RANTES (R&D Systems, Wiesbaden-Nordenstadt, Germany) in EBC were determined using an enzyme-linked immunosorbent assay. The minimum detectable level was 2.0 pg/ml.

Analysis

Statistical significance was analyzed by using analysis of variance (ANOVA) followed by Bonferroni's *t* test post hoc to determine statistical differences. All values were expressed as means ± SD; p values <0.05 were considered significant. The relationship between studied parameters was assayed by correlation. Pearson's linear correlation coefficient was used.

Results

Characteristics of patients and healthy volunteers are presented in Table 1.

Table 1 Characteristics of study subjects and healthy volunteers

Characteristics	Dimension	Healthy volunteers	Stable asthma, steroid naïve	Stable asthma, ICS-treated	Unstable. asthma
Number of patients		7	11	10	12
Sex	F/M	4/3	6/5	6/4	8/4
Age	Years	28.00 ± 4.93	27.36 ± 7.50	44.70 ± 8.11	46.16 ± 5.54
Duration of symptoms	Years		$2.54 \pm 1.21^{b,c}$	$8.60 \pm 3.86^{a,c}$	$15.83 \pm 6.87^{a,b}$
Baseline FEV_1	% Predicted	$106.85 \pm 9.7^{b,c}$	95.63 ± 18.54^{c}	83.60 ± 6.61^{c}	$55.08 \pm 11.91^{a,b}$
Serum total IgE concentration	kU/l	$65.42 \pm 31.65^{a,b,c}$	327.9 ± 265.6	275.0 ± 88.60	316.6 ± 116.2
Blood eosinophil count	cells/mm^3	$51 \pm 26^{a,b,c}$	239 ± 138	271 ± 70	317 ± 112
F_{ENO}	ppB	$18.00 \pm 5.59^{a,b,c}$	76.00 ± 33.66^{b}	$38.70 \pm 10.26^{a,c}$	76.33 ± 27.26^{b}
RANTES (EBC)	pg/ml	$3.56 \pm 0.78^{a,b,c}$	$10.56 \pm 3.01^{b,c}$	$8.18 \pm 1.94^{a,c}$	$20.33 \pm 2.29^{a,b}$
ECP (serum)	μg/l	$3.52 \pm 0.71^{a,b,c}$	12.28 ± 4.02^{c}	11.09 ± 2.87^{c}	$21.46 \pm 9.75^{a,c}$

Data are presented as medians (ranges).
FEV_1, forced expiratory volume in one second.
[a] Values significantly different from patients with stable, steroid-naïve asthma, $p < 0.05$.
[b] Values significantly different from patients with stable, ICS-treated asthma, $p < 0.05$.
[c] Values significantly different from patients with unstable asthma, $p < 0.05$.

In the three groups of asthmatics, EBC concentrations of RANTES were significantly higher than those detected in healthy volunteers (steroid-naïve stable asthma: 10.56 ± 3.01 pg/ml, $p < 0.001$; ICS-treated stable asthma: 8.18 ± 1.94, $p < 0.001$; unstable asthma: 20.33 ± 2.29, $p < 0.001$; healthy volunteers: 3.56 ± 0.78) (Fig. 1). RANTES levels were very strongly elevated in patients with unstable asthma compared with ICS-treated stable asthma patients ($p < 0.001$) and steroid-naïve asthma patients ($p < 0.001$). The concentrations obtained in the steroid-naïve asthma patients group were significantly higher than in the ICS-treated stable asthma group ($p = 0.046$).

We observed statistically significant correlations between the concentrations of RANTES in EBC and F_{ENO} in the three studied groups of asthmatics; these correlations were strong positive in the group of unstable and steroid-naïve stable asthmatics. There were no correlations between RANTES in EBC and F_{ENO} in the group of healthy volunteers. We discovered a significantly positive correlation between RANTES in EBC and serum ECP or blood eosinophil count in the group of asthmatics with severe, unstable asthma and between RANTES and serum ECP in the group of steroid-naïve stable asthmatics. Statistically significant correlations between RANTES in EBC and other studied parameters were not observed in any studied groups of asthmatics or healthy volunteers (Table 2).

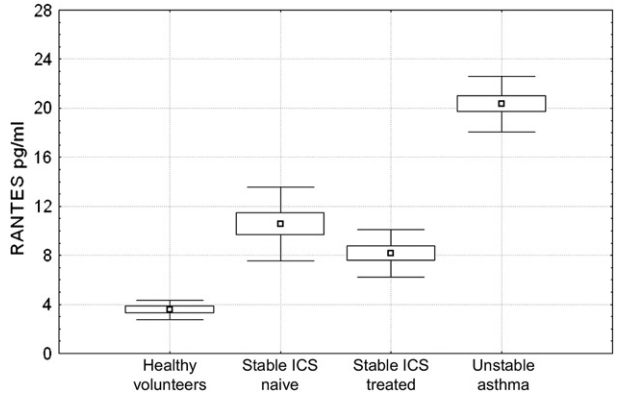

Figure 1 Concentrations of RANTES in EBC in studied groups of asthma patients and healthy volunteers.

Discussion

Eosinophilic infiltration into affected tissue is one of the hallmarks of allergic inflammation. Airway eosinophilia has been demonstrated in asthmatic individuals as compared with normal controls.[11] Airway eosinophilia is a hallmark of asthma exacerbation. Coordination of cytokines such as interleukin-5 (IL-5), eotaxin, and RANTES seems to be necessary for eosinophil extravasation including adhesion, chemotaxis, and activation.[2] *In vitro* studies have demonstrated the specificity of these eosinophil-selective cytokines for attraction and activation of eosinophils, and imply their participation in the specific recruitment of eosinophils to sites of allergic inflammation.[12,13]

RANTES, a member of the Cys-Cys (CC) chemokines, is produced by endothelium cells, fibroblasts, T-lymphocytes, eosinophils, platelets, and other cells.[14] RANTES is a potent chemoattractant for eosinophils, T-lymphocytes, basophils, monocytes/macrophages, and mast cells.[14] It also activates these immune cells and induces the exocytosis of bronchoconstrictive mediators, such as histamine and cysteinyl leukotrienes from basophils and eosinophilic cationic protein from eosinophils.[14] Therefore, RANTES might be involved in inflammatory cell recruitment and the induction of bronchoconstrictive mediators from cells resulting in airflow limitation.

Evidence for an *in vivo* role of RANTES as a chemoattractant in allergic inflammation derives from a study showing that neutralization of RANTES with a receptor antagonist significantly inhibits both lymphocyte and eosinophil recruitment.[15] Teran et al. have reported that RANTES is a major eosinophil attractant in the bronchoalveolar lavage fluid of asthmatic patients exposed to an allergen

Table 2 Correlations between concentrations RANTES in EBC and other studied parameters in the groups of asthma patients and healthy volunteers

Studied groups	F_{ENO}	Serum ECP	Blood eosinophil count	Serum total IgE	Baseline FEV_1
Healthy volunteers	$r = 0.28$	$r = 0.30$	$r = 0.70$	$r = -0.23$	$r = -0.04$
	$p = 0.53$	$p = 0.50$	$p = 0.08$	$p = 0.61$	$p = 0.92$
Stable asthma steroid-naïve	$\mathbf{r = 0.94}$	$\mathbf{r = 0.87}$	$r = 0.43$	$r = 0.13$	$r = -0.15$
	$\mathbf{p < 0.001}$	$\mathbf{p < 0.001}$	$p = 0.18$	$p = 0.27$	$p = 0.65$
Stable asthma ICS-treated	$\mathbf{r = 0.63}$	$r = 0.52$	$r = 0.37$	$r = -0.01$	$r = 0.23$
	$\mathbf{p = 0.048}$	$p = 0.12$	$p = 0.21$	$p = 0.96$	$p = 0.47$
Unstable asthma	$\mathbf{r = 0.93}$	$\mathbf{r = 0.77}$	$\mathbf{r = 0.72}$	$r = 0.41$	$r = -0.35$
	$\mathbf{p < 0.001}$	$\mathbf{p = 0.003}$	$\mathbf{p = 0.008}$	$p = 0.09$	$p = 0.24$

challenge, and the concentration of this cytokine correlates with BAL eosinophil count.[16] Increased RANTES mRNA expression has been demonstrated in bronchial biopsies taken from both allergic and non-allergic asthmatics. The increase in the expression of RANTES mRNA and immunoreactivity at these sites significantly correlated with tissue eosinophil numbers.[17] The levels of this chemokine can be used to predict the development of bronchial hyperreactivity and bronchoconstriction.[15,18]

Corticosteroids are currently the most effective anti-inflammatory agents in the treatment of asthma. Therapy with inhaled corticosteroids leads to a decrease in counts of inflammatory cells (such as eosinophils and T-lymphocytes) and a reduction in the expression of pro-inflammatory cytokines in the airways.[19] Studies performed by Wang et al. have demonstrated that human bronchial epithelial cells are capable of releasing RANTES. This release is significantly attenuated by corticosteroids.[20] John et al. suggest that steroids are effective in reducing airway inflammation in asthma by limiting both RANTES and an IL-8-induced influx of inflammatory cells in the airways.[21]

EBC examination being simple and non-invasive could be exploited to detect specific levels of biomarkers and monitor the severity of disease in response to appropriate prescribed therapy.[9] There is one report concerning the possibilities for measurement of RANTES concentrations in EBC in patients with asthma. Matsunaga et al. have indicated that the level of RANTES in EBC was significantly upregulated in asthmatic airways compared with that of healthy subjects, and that it significantly correlates with the degree of bronchial obturation.[18]

Our study shows that RANTES levels in EBC are higher in asthmatic patients than in healthy controls. In patients with unstable asthma, levels of RANTES were significantly higher than in steroid-naïve and ICS-treated patients with stable asthma.

This observation is confirmed by previous studies performed by Tillie-Leblond et al.[22] They reveal, that CC chemokines (including RANTES) are present at high levels in BAL from patients with severe asthmatic exacerbation; these levels are higher than in patients with mild asthma. The authors suggest that CC chemokines could participate not only in eosinophil recruitment but also in other inflammatory processes associated with severe asthma.

Our results show that, concentrations of RANTES in EBC significantly correlate with exhaled nitric oxide levels — a more and more appreciable criterion for the evaluation of airway inflammation — in the all studied groups.[23] RANTES also correlates with other laboratory tests commonly associated with asthma, such as elevated levels of eosinophil cationic protein (unstable asthma, steroid-naïve stable asthma) and peripheral blood eosinophilia (unstable asthma). We have recorded statistically significant lower levels of RANTES in the group of ICS-treated mild-to-moderate asthmatics compared to steroid-naïve mild allergic asthma patients. This could suggest the beneficial effect of ICS-treatment in down-regulation of RANTES in the airways. However, more studies are needed to establish the influence of ICS on RANTES levels in EBC.

Measurements of RANTES in the EBC of asthma patients may provide another useful diagnostic tool for detecting and monitoring inflammation, disease severity, and responses to treatment.

Competing interests

The authors declare that they have no competing interests in the publication of the manuscript. This work was supported by research grant No 3-35523P from the Medical University of Bialystok, Poland.

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