



Exhaled RANTES and interleukin 4 levels after exercise challenge in children with asthma

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ABSTRACT

Background: Despite the universality and clinical significance of exercise-induced bronchospasm (EIB), the mechanisms responsible for it are incompletely understood.

Objective: To investigate the role of exhaled RANTES (regulated on activation, normal T-cell expressed and secreted) and interleukin (IL) 4 in EIB in children with asthma.

Methods: Fifty-six children with asthma were evaluated with exercise challenge and exhaled RANTES and IL-4 levels. Exhaled breath condensate was collected before and 30 minutes after exercise challenge. RANTES and IL-4 concentrations were determined using a specific immunoassay kit.

Results: A significant increase was found in RANTES levels after exercise challenge in the asthmatic children ($P < .001$). A statistically significant increase in RANTES levels was noted after exercise challenge in both the asthmatic children with EIB ($n = 25$, $P = .007$) and in the non-EIB asthmatic group ($n = 31$, $P = .005$). Our study revealed that exhaled RANTES level correlates significantly well with percentage of forced expiratory volume in 1 second (FEV_1), exacerbation frequency, serum IgE, and body mass index. No statistically significant increase was found in IL-4 levels after exercise challenge. The maximal postexercise decrease in FEV_1 strongly correlated with total eosinophil count ($P < .001$, $r = -0.61$) and baseline ratio of FEV_1 to forced vital capacity (FVC) ($P = .002$, $r = 0.40$). Results from multivariate regression analysis adjusted for age, sex, and atopy as covariates showed that eosinophil count and FEV_1 /FVC ratio were significantly associated with EIB.

Conclusion: We found that exercise challenge, leading to hyperosmolar stimulus, may increase exhaled RANTES levels in children with asthma. In addition, exhaled RANTES levels correlate well with serum IgE, severity of asthma, FEV_1 /FVC ratio, and body mass index. RANTES and IL-4 may not be independent predictors for EIB. Furthermore, eosinophil count and FEV_1 /FVC ratio may predict the presence and severity of EIB in asthmatic children.

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Introduction

Exercise-induced bronchospasm (EIB), a clinical reflection of bronchial hyperreactivity and airway inflammation, is an important feature of asthma that prevents children from living a normal life and decreases their life quality. Despite the universality and clinical significance of EIB, the responsible mechanisms are incompletely understood.¹

There are 2 important hypotheses for EIB: one based on the vascular response to thermal change and the other, more widely accepted osmolar theory, which suggests that dehydration of the airway leads to a hyperosmolar release of mediators from airway mast cells and epithelial cells.^{2–5} In this regard, Furuichi et al⁶ reported that RANTES (regulated on activation, normal T-cell expressed and secreted) and interleukin (IL) 8 are produced by bronchial epithelial cells in response to hyperosmolar stimulation.

The CC chemokines, such as RANTES and eotaxin, are believed to be involved in the pathogenesis of asthma by promoting the migration and activation of inflammatory cells, especially eosinophils.⁷ An increased expression of local and systemic CC chemokines has been demonstrated in asthmatic patients in studies performed using bronchoalveolar lavage (BAL), induced sputum,

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and exhaled breath condensate (EBC).^{8–11} Furthermore, there is limited knowledge about the role of exhaled IL-4, a cytokine for the T_H2 phenotype, in asthma.^{12,13} In contrast to BAL and induced sputum, EBC is an entirely noninvasive method for the collection of airway secretions.^{14,15}

It is crucial to clarify the pathogenesis of EIB to assist in the diagnosis and treatment of asthma. As proof of the osmotic theory, showing an increase in RANTES levels in the airways and investigating the role of T_H2 response by IL-4 measures in the airway secretions are inspiring topics for clarifying the EIB pathogenesis. To our knowledge, no previous study has investigated the role of exhaled RANTES and IL-4 levels in EIB in children with asthma.

The primary aim of the present study was to determine RANTES and IL-4 levels and their variation after exercise challenge in the EBC of asthmatic children and to establish probable correlations of these measurements with other parameters. The secondary outcome of our study was to determine the factors that designate EIB positivity and severity.

Methods

Study participants

Fifty-six asthmatic children (39 boys and 17 girls) who sought care at the Gaziantep University Pediatric Allergy and Asthma Unit from September 2010 to April 2011 were included in our study. The children were 6 to 18 years old and had asthma according to the criteria described by the Global Initiative for Asthma.¹⁶ All patients had been in a stable condition and free from acute exacerbations and respiratory tract infections in the previous 2 months and had no systemic diseases other than asthma. Twenty-six children with mild-to-moderate persistent asthma had been undergoing maintenance therapy with low-to-medium constant doses of inhaled corticosteroids (budesonide or fluticasone) for at least 2 months. Thirty children were free of steroids. None of the patients was being treated with leukotriene receptor antagonists. Asthmatic children used β_2 -agonists on demand, but they refrained from taking this medication for at least 24 hours before the test.

Study design

The child's family completed a questionnaire concerning EIB, the nature and duration of asthma symptoms, and medication use. Before the exercise test, total eosinophil and immunoglobulin IgE levels were assessed, and skin prick tests to commonly encountered aeroallergens (house dust mites, trees, weeds, grasses, cat, *Alternaria*, *Cladosporium*, egg white, cow's milk) and forced expiratory volume in 1 second (FEV₁) were performed in each asthmatic child.

The asthmatic children enrolled in the study underwent physical examination and EBC collection and then completed a standardized treadmill exercise test. The EBC was collected before and 30 minutes after the exercise test.

The study was performed as a prospective trial. The personnel performing the enzyme-linked immunosorbent assay (ELISA) tests were unaware of the clinical status of the case or protocol of the study. The primary outcome of the study was the change in EBC RANTES and IL-4 levels after exercise test and the relation with EIB.

Exercise challenge and spirometry

Exercise testing was performed on an electrically driven treadmill for 6 minutes with patients wearing a nose-clip under stable environmental conditions. The workload was increased until 80% of the predicted maximal heart rate was reached. All patients were studied in the morning.

Spirometric measurements were performed with patients at rest and 1, 5, 10, 15, and 20 minutes after the end of exercising by using a dry spirometer (Vmax22; Sensor Medics, Homestead,

Florida). The best 3 FEV₁ values were expressed as a percentage of predicted reference values. To investigate patients with a significant decrease in FEV₁ after exercise, a 10% postexercise decrease in FEV₁ cutoff was considered suitable because this is generally accepted to define EIB. Heart rate and oxygen saturation were recorded continuously with a pulse oximeter during exercise.

Collection of EBC

EBC was collected (ECoScreen; Jaeger, Hochberg, Germany) according to the American Thoracic Society/European Respiratory Society Task Force recommendations.¹⁷ All patients breathed in a relaxed manner (tidal breathing) for 15 minutes not wearing a nose-clip. Approximately 1 mL of breath condensate was collected in sterile Eppendorf tube and was immediately stored at –80°C for later analysis.

Measurements of EBC

Exhaled RANTES and IL-4 concentrations were quantified using a specific enzyme immunoassay kit (Ray Biotech, Norcross, Georgia, and Biosource, Nivelles, Belgium, respectively). The detection limits for RANTES and IL-4 were 3 and 1.2 pg/mL, respectively.

Ethics

All study procedures were performed in accordance with a protocol previously approved by the Ethics Committee of Gaziantep University. All parents provided written informed consent for the study procedures, and the children gave their assent.

Statistical analyses

A statistical software package was used for all data analysis and comparisons (SPSS statistical software, version 11.5; SPSS Inc, Cary, North Carolina). Data concerning patient characteristics are expressed as median and interquartile range (IQR). The data were tested for assumptions of normality, and differences between the groups were compared with the Mann-Whitney test. Spearman rank correlation coefficient was applied to investigate the correlation among different parameters. $P < .05$ was considered significant.

Sample size was estimated using a power calculation based on 50% increase in RANTES and IL-4 measurements. It was estimated that at least 12 patients would be required to detect a significant difference at 80% power level and an α error of .05.

Results

Fifty-six consecutive asthmatic children were tested. Characteristics of the asthmatic children are listed in Table 1. Twenty-five asthmatic children had a decrease in FEV₁ of 10% or greater after the exercise, whereas 31 patients had a less than 10% decrease.

EBC RANTES

Exhaled RANTES levels were measured in 75% of the patients. At baseline, no significant difference in EBC RANTES levels was found between the EIB group and the non-EIB asthmatic group (2.7 pg/mL [IQR, 1.9–3.9 pg/mL] vs 2.3 pg/mL [IQR, 1.5–3.0 pg/mL]; $P = .10$) (Table 1).

A statistically significant increase was found in the concentration of RANTES levels after exercise challenge in the asthmatic children (before exercise: 2.6 pg/mL [IQR, 1.8–3.3 pg/mL]; 30 minutes after exercise: 3.6 pg/mL [IQR, 2.7–7.3 pg/mL]; $P < .001$). A statistically significant increase in the concentration of RANTES levels was noted after exercise challenge in the asthmatic children with EIB (before exercise: 2.7 pg/mL [IQR, 1.9–3.9 pg/mL]; 30 minutes after exercise: 4.1 pg/mL [IQR, 3.1–8.1 pg/mL]; $P = .007$; Fig 1)

Table 1
Main characteristics of the study population at baseline

Characteristic	EIB children (n=25)	Non-EIB children (n=31)	P value
Male/female, No.	21/4	18/13	.03
Age, mean (SD), y	11.5 (2.1)	11.2 (2.1)	.34
Duration of asthma, mean (SD), y	4.3 (3.5)	4.2 (3.8)	.96
Atopy, %	78	51	.04
Treatment			.03
Regular use of ICSs, %	56	40	
No regular treatment	44	60	
FEV ₁ , median (IQR), % predicted	85.0 (76.0–92.0)	91.0 (85.0–99.0)	.03
FEF _{25%–75%} , median (IQR), % predicted	68.0 (51.0–83.0)	86.0 (70.0–100.0)	.01
FEV ₁ /FVC, median (IQR), % predicted	86.0 (79.0–89.0)	92.0 (85.0–97.0)	.002
Maximum FEV ₁ decrease, median (IQR), %	–22 (–32 to –14)	–3 (–6 to 0)	<.001
EBC RANTES, median (IQR), pg/mL	2.7 (2.0–3.9)	2.3 (1.5–3.0)	.10
EBC IL-4, median (IQR), pg/mL	184.9 (151.4–238.6)	184.5 (151.8–246.1)	.99
IgE, median (IQR), IU/L	270.0 (156.0–768.0)	68.2 (25.2–363.0)	.008
Eosinophil, median (IQR), %	6.7 (5.4–8.5)	2.6 (1.2–4.3)	<.001

Abbreviations: EBC, exhaled breath condensate; EIB, exercise-induced bronchospasm; FEF_{25%–75%}, forced expiratory flow, midexpiratory phase; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; ICSs, inhaled corticosteroids; IL-4, interleukin 4; IQR, interquartile range.

and without EIB (2.3 pg/mL [IQR, 1.5–3.0 pg/mL] vs 3.5 pg/mL [IQR, 1.4–6.9 pg/mL]; $P=.005$; Fig 1).

Factors that affect exhaled RANTES levels

There was a significant negative correlation between preexercise RANTES levels and baseline FEV₁ ($P=.03$, $r=-0.33$, Spearman rank correlation coefficient, Fig 2). Preexercise RANTES levels strongly correlated with total IgE and body mass index (BMI) ($P=.006$, $r=0.42$ and $P=.04$, $r=0.42$, respectively, Fig 2). Postexercise RANTES levels strongly correlated with asthma exacerbation per year ($P=.01$, $r=0.57$, Spearman rank correlation coefficient, Fig 2) and preexercise RANTES levels ($P=.001$, $r=0.41$). No significant correlation was found between maximal postexercise decrease in FEV₁ and exhaled RANTES levels.

EBC IL-4

Exhaled IL-4 levels were detected in 100% of the patients. At baseline, no significant difference in EBC IL-4 levels was found between the EIB group and the non-EIB asthmatic group (184.9 pg/mL [IQR, 151.4–238.6 pg/mL] vs 184.5 pg/mL [IQR, 151.8–246.1 pg/mL]; $P>.05$) (Table 1). No statistically significant increase was found in the concentration of IL-4 levels after exercise challenge in the asthmatic children with or without EIB (200.0 pg/mL [IQR,

154.9–236.9 pg/mL] vs 209.0 pg/mL [IQR, 170.0–264.0 pg/mL]). When we assessed the influence of inhaled corticosteroid treatment on exhaled mediators, we could not find any relation between treatment and exhaled mediators evaluated before and after exercise challenge (data not shown).

Relative risks for EIB obtained by logistic regression analysis

Univariate analysis identified eosinophil count, sex, skin test positivity, ratio of FEV₁ to forced vital capacity (FVC), percentage of FEV₁, and forced expiratory flow, midexpiratory phase (FEF_{25%–75%}) as significantly associated with EIB (Table 2). Results from the multivariate regression analysis adjusted for age, sex, and atopy as covariates revealed that eosinophil count and FEV₁/FVC ratio were significantly associated with EIB (Table 2).

Factors that affect severity of EIB

The maximal postexercise decrease in FEV₁ strongly correlated with baseline FEV₁/FVC ratio and total eosinophil counts ($P=.002$, $r=0.40$ vs $P<.001$, $r=-0.61$; Fig 3). The maximal postexercise decrease in FEV₁ strongly correlated with total IgE levels and baseline FEF_{25%–75%} ($P=.007$, $r=-0.37$ vs $P=.03$, $r=0.29$).

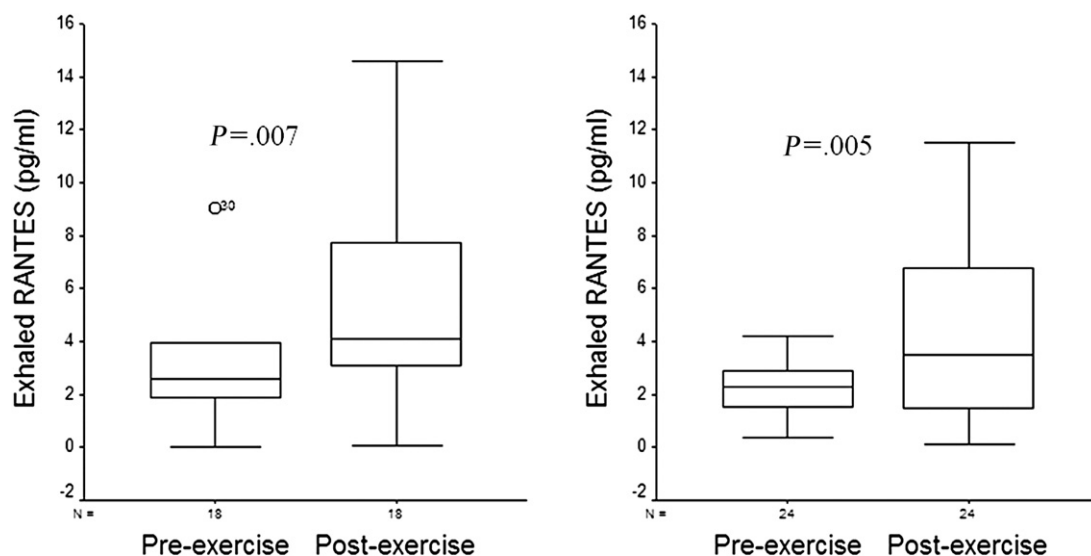


Figure 1. Median exhaled RANTES measures before and after exercise challenge in children with exercise-induced bronchospasm (left) and without exercise-induced bronchospasm (right). Error bars indicate interquartile ranges.

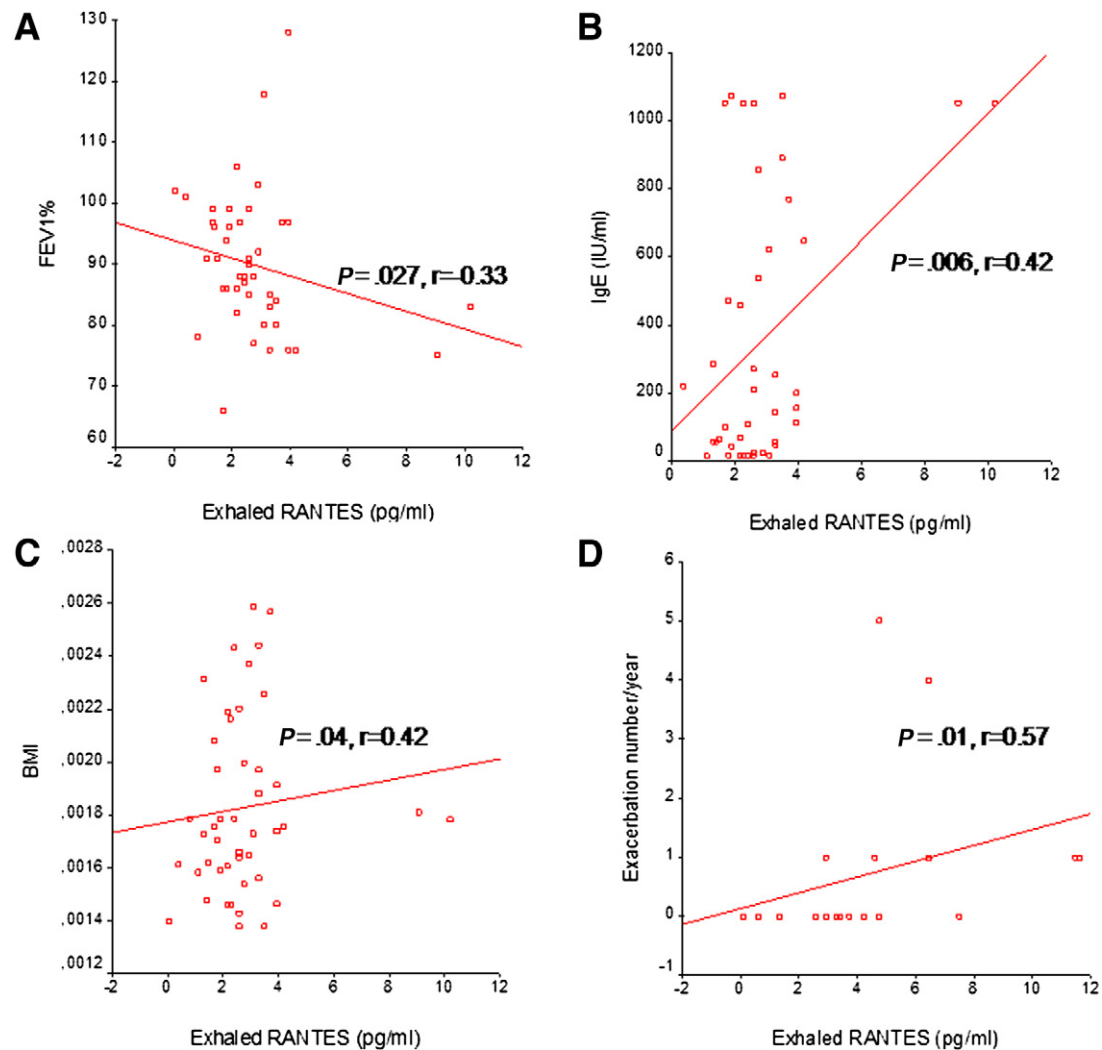


Figure 2. Correlation between exhaled RANTES and forced expiratory volume in 1 second (FEV₁) levels (A), total IgE measures (B), body mass index (BMI) (C), and exacerbation frequency (D) in children with asthma.

Relationship between atopy and exhaled mediators

Significant differences were found between *Alternaria*-sensitive and *Alternaria*-insensitive children in EIB severity (-18 [IQR, -35 to -5] vs -6 [IQR, -14 to -1]; $P=.02$), eosinophil percentage (6.8 [IQR, 4.2 – $8.$] vs 3.1 [IQR, 1.4 – 5.8]; $P=.005$), and postexercise IL-4 levels (236 [IQR, 212 – 297] vs 175 [IQR, 147 – 227]; $P=.02$). A marginally significant difference was found between *Alternaria*-sensitive and *Alternaria*-insensitive children in the percentage of

RANTES change after exercise challenge (131 [IQR, 95 – 229] vs 62 [IQR, -16 to 173]; $P=.09$).

Discussion

EIB, a good indicator of bronchial hyperreactivity and airway inflammation, is observed in 70% to 80% of asthmatic patients^{18,19} and is an important and debilitating manifestation of asthma. By preventing children from participating in normal daily activity, EIB

Table 2

Relative risks for EIB obtained by univariate and multivariate logistic regression analysis of various factors in asthmatic children

Factor	Univariate		Multivariate	
	OR (95% CI)	P value	OR (95% CI)	P value
Age	1.0 (0.98–1.03)	.47	1.004 (0.97–1.03)	.78
Female sex	0.26 (0.07–0.95)	.04	0.59 (0.12–2.97)	.53
Skin test positivity	3.38 (1.001–11.38)	.05	1.05 (0.21–5.2)	.95
Eosinophil count	1.48 (1.18–1.85)	.001	1.39 (1.08–1.78)	.01
FEV ₁ /FVC ratio	0.88 (0.81–0.96)	.004	0.89 (0.81–0.98)	.03
FEF _{25%–75%}	0.96 (0.94–0.99)	.01		
Preexercise RANTES measure	1.50 (0.9–2.6)	.10		
IgE	1.002 (1.001–1.003)	.06		

Abbreviations: CI, confidence interval; FEF_{25%–75%}, forced expiratory flow, midexpiratory phase; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; NS, not significant; OR, odds ratio.

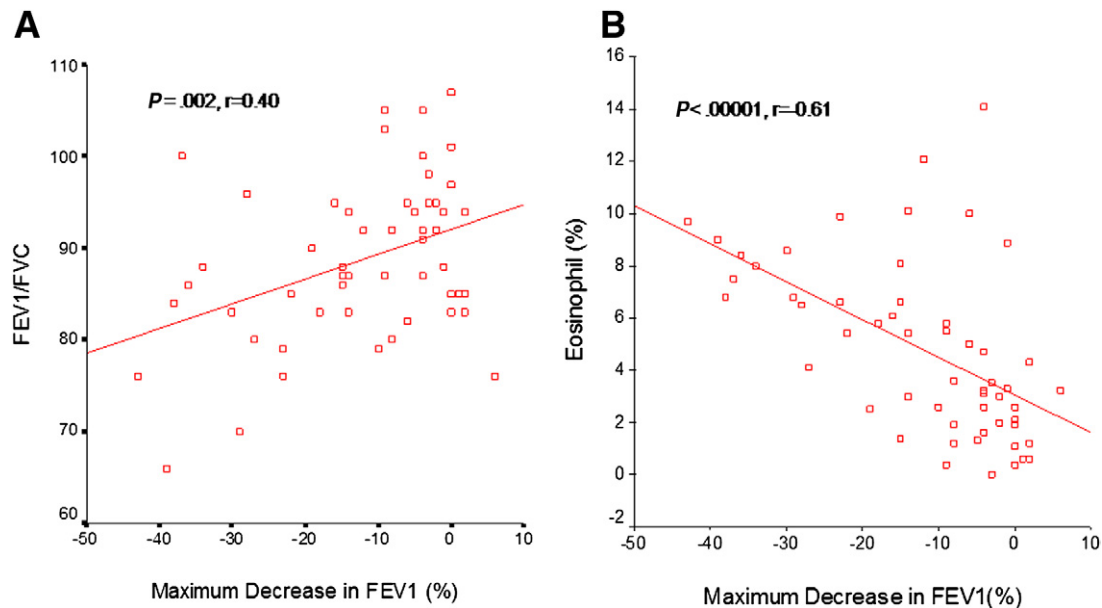


Figure 3. (A) Correlation between maximum forced expiratory volume in 1 second (FEV_1) decrease after exercise challenge and ratio of FEV_1 to forced vital capacity (FVC). (B) Correlation between maximum FEV_1 decrease after exercise challenge and eosinophil percentage.

disrupts their life quality, and its presence categorizes the patient in the partially controlled or uncontrolled asthma categories.

Although the pathogenesis of EIB is not completely understood, it has been suggested that hyperosmolarity caused by cooling and drying of the airway mucosa during exercise results in mast cell degranulation and release of mediators.¹ However, the evidence related to the contribution of inflammatory mediators in either the induction or maintenance of bronchoconstriction induced by exercise is contradictory.²⁰ To our knowledge, no study has investigated the role of exhaled RANTES and IL-4 levels in EIB in children with asthma.

Evaluating the role of exhaled RANTES and IL-4 in EIB, we found that, regardless of the outcome of exercise testing, exhaled RANTES levels increase in asthmatic children after exercise challenge. Comparable to our finding, Furuichi et al⁶ found in an *in vitro* study that RANTES and IL-8 are produced by bronchial epithelial cells in response to hyperosmolar stimulation. Concordantly, the hyperosmolar stimulus generated by exercise provocation may enhance RANTES secretion from bronchial epithelial cells, leading to an increase in exhaled RANTES measures. RANTES, a member of the CC chemokines, is a powerful chemoattractant and activator of eosinophils, T lymphocytes, and basophils, inducing the exocytosis of mediators,²¹ which may play an important role in the upregulation of airway inflammation. Because we could not demonstrate any relation between EIB severity and increase in exhaled RANTES levels, RANTES may not be an independent predictor for EIB. To interpret, exercise challenge leading to hyperosmolar stimulus may increase exhaled RANTES levels even in asthmatic children without EIB, suggesting that, although RANTES was secreted from bronchial epithelium after exercise challenge, other mediators or mechanisms might contribute collectively in the emergence of EIB. Capability of the patient to secrete cysteinyl leukotrienes or adenosine and the sensitivity of the airway smooth muscle may be involved in the determination of bronchoconstriction.^{22–25} It has previously been reported that exhaled cysteinyl leukotriene measures are higher in children with EIB compared with non-EIB children with asthma.²² In addition, Csoma et al²³ demonstrated that exhaled adenosine levels increase after exercise challenge, and this increase was correlated with the decrease in FEV_1 values.²³

Similar to our study, Zietkowski et al²⁶ demonstrated that there was a statistically significant increase in the concentration of ex-

haled RANTES in asthmatic adults with EIB. In contrast to our finding, they reported that exercise did not affect the concentrations of exhaled RANTES in asthmatic adults without EIB. However, as they mentioned, the small number of asthmatic adults without EIB may have played a role in this controversial result.

EBC has proven to be a simple, noninvasive, and safe technique for use in children,^{27,28} and it can also be repeated several times, enabling exhaled markers of airway inflammation to be longitudinally monitored²⁹ without affecting the airway, in contrast to bronchial biopsy, BAL, and induced sputum.

Our study found that exhaled RANTES level correlates well with FEV_1 , number of exacerbations in the last year, serum IgE level, and BMI. This correlation may be indicative of a link among pulmonary obstruction, severity of disease, atopy, BMI, and inflammation in the airways of children with asthma. As in our study, Matsunaga et al¹¹ reported a significant relation between RANTES and percentage of FEV_1 in children with asthma. Concordantly, a previous report found that RANTES-positive sputum eosinophils and the percentage of FEV_1 after allergen challenge are significantly correlated in asthmatic patients.³⁰ We also proved that when the exacerbation frequency increases, exhaled RANTES increases in asthmatic children. Similarly, Zietkowski et al¹⁰ reported that exhaled RANTES levels are higher in unstable compared with stable asthma. As indicated in our study, there was a positive correlation between serum IgE and exhaled RANTES; Redhu et al³¹ further found that IgE sensitization of bronchial and tracheal smooth muscle cells significantly enhanced the transcription of RANTES at the promoter level. On the other hand, Kimata et al³² demonstrated that RANTES enhanced IgE production by directly stimulating surface IgE-positive B cells. In light of these results, there may be a reciprocal relation between RANTES and IgE. There was also a remarkable positive relation between RANTES and BMI in our study, concordant with the survey by Herder et al.³³ In support of our result, it has been substantiated that RANTES is upregulated in white adipose tissue of obese compared with lean individuals³⁴ and cord blood RANTES is selectively elevated with fetal macrosomia.³⁵

It is also well known that IL-4, as a T_H2 phenotype cytokine, may be involved in the pathogenesis of asthma.^{13,36,37} On the other hand, the relationship between EIB and IL-4 is not fully understood. No significant increase in IL-4 levels was found after exercise challenge in asthmatic children in our study. IL-4 may not be an inde-

pendent predictor for EIB. To our knowledge, no previous study has searched the relation between exhaled IL-4 and EIB.

When we search the factors that designate EIB positivity, multivariate analysis identified eosinophil percentage and FEV₁/FVC ratio as predictive of EIB positivity. In addition, a strong correlation was found between eosinophil percentage and FEV₁/FVC ratio and EIB severity. By evaluating eosinophil percentage and FEV₁/FVC ratio, physicians may estimate the presence and the severity of EIB and may decide on the treatment of the asthmatic patients. In this regard, it was found that although baseline pulmonary function in EIB asthmatic patients did not differ from those without EIB, FEV₁ and FEF_{25%–75%} were significantly correlated with EIB severity.³⁸ Furthermore, it has been demonstrated that eosinophils produce and release cysteinyl leukotrienes in connection with hyperosmolar stimulus, which may cause EIB.^{39,40} Concordant with our result, Lee et al³⁸ demonstrated that blood eosinophilia is an important determinant in the severity of EIB.

A limitation of our study is that a group of healthy individuals was not included in the study design as controls. However, Zietkowski et al²⁶ demonstrated previously that exercise did not affect the concentrations of exhaled RANTES in healthy adults. Our results also support the in vitro study that reported that RANTES is produced by bronchial epithelial cells in response to hyperosmolar stimulation.⁶ Furthermore, it is known that intense exercise may cause airway injury and EIB in elite athletes. Additional studies investigating exhaled RANTES levels in response to exercise challenge in healthy athletes compared with asthmatic athletes are necessary.

In conclusion, although RANTES may not be an independent predictor for EIB, exercise challenge, leading to hyperosmolar stimulus, may increase exhaled RANTES levels, promoting the migration and activation of inflammatory cells, including eosinophils, and thereby having an important role in the upregulation of airway inflammation in children with asthma. In addition, exhaled RANTES level correlates well with serum IgE, asthma severity, pulmonary function, and BMI. Furthermore, eosinophil count and pulmonary function measurements may predict the presence and severity of EIB in asthmatic children.

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