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The roles of a Th2 cytokine and CC chemokine in children with stable asthma: Potential implication in eosinophil degranulation

Chang K. Kim¹, Hirohito Kita², Zak Callaway¹, Hyo B. Kim¹, Jungi Choi¹, Takao Fujisawa³, Bo M. Shin⁴, and Young Y. Koh⁵

¹Pediatric Asthma and Allergy Center, Inje University Sanggye Paik Hospital, Seoul, Korea

²Department of Immunology, Mayo Clinic and Foundation, Rochester, USA

³Department of Pediatrics, Mie National Hospital, Tsu, Japan

⁴Department of Clinical Pathology, Inje University Sanggye Paik Hospital, Seoul, Korea

⁵Department of Pediatrics, Seoul National University Hospital, Seoul, Korea

Abstract

Th2 cytokine IL-5 and CC chemokine eotaxin are thought to be key regulators of eosinophils in bronchial asthma. However, their involvement in children with stable asthma (SA) has not been determined. We investigated the roles of IL-5 and eotaxin in eosinophil degranulation in children with SA. Induced sputum was obtained from 30 SA, 21 allergic rhinitis (AR), and 22 non-atopic healthy control (HC) children. We measured sputum levels of IL-5, eotaxin, and eosinophil indices [percentage eosinophils, eosinophil-derived neurotoxin (EDN), and eosinophil cationic protein (ECP)]. We also examined correlations of IL-5 and eotaxin with eosinophil indices. Sputum percentage eosinophils and EDN and ECP levels were significantly higher in the SA group than in the HC group, while only the sputum EDN and ECP levels were significantly higher in the AR group than in the HC group. Unexpectedly, sputum levels of IL-5 were not significantly different among the three groups; however, the levels of eotaxin were higher in the SA group when compared to the HC group. No significant correlations were found between IL-5 and percentage eosinophils, EDN, or ECP levels; in contrast, eotaxin levels correlated significantly with percentage eosinophils ($R = 0.638$; $p = 0.0001$), EDN ($R = 0.522$; $p = 0.003$), and ECP levels ($R = 0.630$ and $p = 0.0002$). The elevated levels and good correlations of eotaxin with sputum eosinophil indices, and no elevation or correlation of IL-5 with these indices, suggest that CC chemokine eotaxin may play a more important role in eosinophil degranulation in children with SA.

Keywords

eosinophil degranulation; eosinophil indices; eotaxin; interleukin-5; stable asthma

Eosinophils play a central role in the inflammatory response associated with bronchial asthma (1). The recruitment of eosinophils into sites of airway inflammation is a complex process that potentially is regulated by various cytokines, including interleukin (IL)-3, IL-5, and granulocyte macrophage colony stimulating factor (GM-CSF), and by various chemokines, including eotaxin, regulated on activation, normal T-cell expressed and

secreted (RANTES), and monocyte chemoattractant protein-3 (MCP-3) (2). Cooperation of the eosinophilic cytokines and chemokines seems to be necessary for eosinophil recruitment in the airways (3). The striking feature of the eosinophilic inflammatory reaction is the marked deposition of eosinophil degranulation products, such as eosinophil-derived neurotoxin (EDN) and eosinophil-cationic protein (ECP), and has been documented in asthma (4). However, the precise mechanisms of eosinophil degranulation *in vivo* are still poorly understood.

An increase in Th2 cytokine production has been found in asthma. The cytokine IL-5, a key Th2 cytokine in eosinophil biology, is involved in eosinophil differentiation, maturation, and migration. IL-5 has been shown to be chemotactic for eosinophils *in vitro* (5). Indeed, an increase in IL-5 production in the airways has been found in acute exacerbation of asthma (6). However, the role of IL-5 in human asthma is not as strong as previously suspected and is currently being reevaluated. Flood-Page et al. (7) observed in an allergen-challenged asthma model that anti-IL-5 treatment produced a weak reduction in airway eosinophils and no change in the level of eosinophilic degranulation. The results from the allergen-challenged asthma model and acute asthma exacerbation do not necessarily reflect events in the natural course of asthma (high dose vs. low dose of allergen exposure). Furthermore, in pediatric asthma, the role of IL-5 in eosinophilic airway inflammation is not fully understood. Several CC chemokines, such as eotaxin, RANTES, and MCP-3, have been implicated in eosinophil recruitment and degranulation. Eotaxin seems specific for eosinophil chemoattraction (8); however, RANTES and MCP-3 are active on eosinophils as well as other leukocytes (2). CC chemokines bind to a CCR3 receptor, a chemokine receptor highly expressed on eosinophils, suggesting their key role in eosinophilic inflammation (9).

The aim of this study was to evaluate the production of IL-5, the predominant eosinophil-active Th2 cytokine (10), and eotaxin, the most important eosinophil chemoattractive CC chemokine (5), in airways of children with stable asthma (SA). We also examined correlations of IL-5 and eotaxin with eosinophil indices (percentage eosinophils and EDN and ECP levels).

Methods

Subjects

Induced sputum was obtained from 30 SA, 21 allergic rhinitis (AR), and 22 non-atopic healthy control (HC) subjects. At the time of the study, the patients with asthmatic had SA, with no acute asthma symptoms, nor evidence of chest infection. Eleven out of 21 AR subjects had seasonal AR. All subjects had not taken oral glucocorticoids for at least 8 wk prior to the study.

Spirometry was performed, and baseline forced expiratory volume in 1 s (FEV₁) was expressed as the best of three reproducible values with a maximal change of 5%. Methacholine challenge tests were interpreted as a positive result if a 20% reduction in FEV₁ following inhaled methacholine was noted. Allergy skin prick tests were performed, and atopy was defined as having at least one positive skin prick reaction to a battery of 12 common aeroallergens in Korea.

The SA group consisted of 17 boys and 13 girls, 7–15 yr of age (mean, 9.2 yr). All patients in this group were atopic and had been given a diagnosis of asthma on the basis of previous asthma symptoms with proven β_2 -agonist reversibility and/or a positive methacholine challenge. A total of 18 out of the 30 patients with asthmatic were taking inhaled corticosteroids (ICS), while the remaining nine were steroid-naïve asthmatics.

The AR group consisted of 12 boys and 9 girls, 7–13 yr of age (mean, nine yr). Inclusion criteria included having mainly eye and nose allergic symptoms, normal methacholine responsiveness, and a normal chest radiograph.

The HC group consisted of 10 boys and 12 girls, 8–15 yr of age (mean, 10.4 yr). None of them had a history of asthma or allergic disease. They had no identifiable airway infections in the 4 wk prior to the study. They had normal expiratory flows (an FEV₁ > 80% predicted) and methacholine responsiveness.

Parents of the patients and the control subjects gave informed consent, and the study was approved by the Hospital Ethics Committee.

Sputum induction and processing

All sputum inductions in patients with AR were carried out during the winter season to minimize the use of intranasal corticosteroids. All subjects were pre-medicated with two puffs of albuterol. Sputum induction was performed with aerosolized 3% hypertonic saline generated by a DeVilbiss Ultra-Neb 99 ultrasonic nebulizer (the DeVilbiss Co, Somerset, PA, USA), as described by Fahy et al. (11), but modified.

Subjects were asked to rinse their mouth with water, swallow, and blow their nose to minimize contamination of sputum with saliva and post-nasal drip. Every 2 min for up to 12 min, they were instructed to cough to produce sputum into a sterile container. For all subjects, FEV₁ was monitored 15 min post-albuterol and at 4-min intervals thereafter throughout the test for safety. The collected sputum was processed, as described by Pizzichini et al. (12), but modified. Sputum was processed within 2 h of collection. Equal volumes of sputum were placed into two 15-ml polypropylene tubes. One aliquot was treated with an equal volume of saline solution containing 0.1% dithiothreitol (DTT) (1:10 diluted DTT) (Sputolysin 1%; Calbiochem Corp., San Diego, CA, USA) ('with DTT' aliquot). The other aliquot was mixed with twice the volume of saline solution with no DTT ('without DTT' aliquot). Both mixtures were aspirated up and down with a Pasteur pipette, then vortexed for 15 s, and placed on a bench rocker for 20 min. Both aliquots, with and without DTT, were filtered through 40- μ m strainers (Becton Dickinson, Becton Drive Franklin Lakes, NJ, USA) and then centrifuged at 833 g for 10 min at 4°C. The supernatants from only the 'without DTT' aliquots were frozen at -70°C for later assays. Cell pellets from only the 'with DTT' aliquots were resuspended in saline solution and stained with trypan blue, and a total non-squamous cell count was performed with a hemocytometer. Cytospin preparations were adjusted to equal approximately 1.0×10^6 /ml and then treated with Diff-Quik stain (Dade Behring Inc., Newark, DE, USA) for 400 differential non-squamous cell counts. The specimen was considered adequate if morphologic identification of total and differential cell counts could be obtained (13).

Fluid-phase measurements

The levels of IL-5 (Endogen, Woburn, MA, USA) and eotaxin (R&D Systems, Minneapolis, MN, USA) were measured in the thawed supernatant using sandwich ELISAs according to the respective manufacturers' protocols, and the results were expressed in pg/ml. The sensitivity of IL-5 was <2.0 pg/ml and that of eotaxin was less than 5.0 pg/ml. The levels of human EDN and ECP (MBL, Woburn, MA, USA) were measured in the thawed supernatant using sandwich ELISA according to the manufacturer's protocols, with minimum detection limits of 0.62 and 0.125 ng/ml, respectively. The result was adjusted for the dilution. When the cytokine and chemokine levels were below the sensitivity, 0 level was used for level comparisons and 1/2 level of the sensitivity was used for correlations.

Statistical analysis

Cellularity data were expressed as a percentage of the total cell count (cell differential) and were presented as medians with interquartile ranges (25th–75th). Fluid-phase marker levels were expressed per ml and presented as mean $\pm$ s.d. As the majority of data was not normally distributed, non-parametric analysis was used. Screening of data for differences in cell profiles and fluid-phase markers among the three groups was performed using the Kruskal–Wallis test. When significant differences were identified, individual groups were compared using the Mann–Whitney *U*-test. Correlations were analyzed by the Pearson correlation test. A *p* value of <0.01 was considered statistically significant.

Results

Subject characteristics

Subject characteristics are shown in Table 1. All subjects tolerated sputum induction with no adverse effects. Four subjects were excluded from the study: three owing to poor sputum induction (one from the SA group and two from the HC group) and one owing to low FEV₁ percentage predicted (from the SA group). There were no statistical differences between the three groups in terms of age, sex, or FEV₁ percentage predicted. However, blood eosinophils were significantly higher ($p < 0.001$) in the SA and AR groups when compared to the HC group.

Sputum eosinophil indices

Sputum eosinophil percentage and EDN and ECP levels for all study groups are summarized in Table 1.

Sputum eosinophil percentage—Eosinophil percentages in the SA group were significantly higher than those in the HC group ($p < 0.01$), while there was no statistical difference between the AR and HC groups.

Sputum EDN and ECP profiles—EDN levels in the SA group were significantly higher than those in the AR ($p < 0.01$) and HC groups ($p < 0.0001$). ECP levels in the SA group were significantly higher than in the HC group ($p < 0.0001$). ECP levels in the AR group were also significantly higher than in the HC group ($p < 0.001$).

Comparing subgroups (steroid vs. steroid-naïve) based on ICS use, we found no significant differences in percentage eosinophils, EDN, or ECP levels.

Cytokine and chemokine profiles

Comparisons of Th2 cytokine IL-5 and CC chemokine eotaxin levels in the three groups are shown in Fig. 1a,b, respectively. Unexpectedly, no statistically significant differences were observed in the sputum levels of IL-5 among the three groups. Only the SA group (108.8 ± 73.7 pg/ml) (mean $\pm$ s.d.) showed significantly higher ($p < 0.001$) eotaxin levels compared to the HC group (40.3 ± 35.1 pg/ml). Eotaxin levels in the SA group were higher than in the AR group, although it was not statistically significant ($p = 0.058$). No other significant differences in eotaxin levels were found between the three groups.

Correlations

Correlations of IL-5 and eotaxin with sputum eosinophil indices (percentage eosinophils and EDN and ECP levels) in the SA group are shown in Fig. 2a–f. The levels of IL-5 showed no correlations with sputum percentage eosinophils, EDN, or ECP. However, the levels of

eotaxin correlated significantly with percentage eosinophils ($R_s = 0.638$; $p = 0.0001$), EDN ($R_s = 0.522$; $p = 0.003$), and ECP levels ($R_s = 0.630$ and $p = 0.0002$).

When comparing eosinophil indices with IL-5 and eotaxin in the steroid subgroup, IL-5 did not correlate, whereas eotaxin did. Therefore, regardless of steroid use, eotaxin correlated well with eosinophil indices.

There were no significant correlations between IL-5 or eotaxin levels and FEV₁ percentage predicted (data not shown). IL-5 levels did not correlate significantly with eotaxin levels, either (data not shown). The preceding analyses used data from only the SA group.

Discussion

To our knowledge, this is the first report on the roles of the Th2 cytokine IL-5 and CC chemokine eotaxin in the eosinophil degranulation that occurs in pediatric asthma. Our results showed sputum eotaxin levels were increased along with eosinophil indices (percentage eosinophils and EDN and ECP levels) in patients with SA when compared to normal controls. In contrast, sputum IL-5 was not significantly higher. Our results also showed that eotaxin levels correlated positively with percentage eosinophils and the sputum eosinophil degranulation products EDN and ECP. As expected, the levels of IL-5 did not correlate significantly with percentage eosinophils, EDN, or ECP levels. These findings suggest that the CC chemokine eotaxin may play a key role in the degranulation of airway eosinophils in children with SA.

The role of IL-5 in pediatric asthma requires discussion. The 'Th2 hypothesis for asthma' purports that asthma is caused by a relative increase in Th2-cell products (e.g., IL-5 and IL-13) in combination with a decrease in Th1-cell products (e.g., IFN- γ) (14, 15). However, more recent studies suggest that the 'Th2 hypothesis for asthma' is far too simplistic (16). In our study, there were no significant increases in the Th2 cytokine IL-5 in the SA group. In an animal study, IL-5 needed other mediators to induce eosinophil migration from the bone marrow into the lungs (17). The role of IL-5 has also been evaluated in humans.

In a study of asthmatics, anti-IL-5 treatment had no effect on the level of sputum eosinophil degranulation and produced no significant changes in clinical measures of asthma (airway hyper-responsiveness and peak flow recordings) (7). It was proposed that although IL-5 is important in eosinophil development, activation, and survival, other cytokines, such as IL-3 and GM-CSF, may sustain tissue eosinophilia in its absence. Liu et al. (18) observed that eosinophil recruitment to the airway lumen may be regulated in part by factors other than IL-5. Furthermore, Liu et al. (19) suggested that when eosinophils are recruited to the airway, regulation of their function becomes IL-5-independent based on their observation of the loss of membrane IL-5 receptor alpha. A recent study of adult SA tics by Xu et al. (20) demonstrated IL-5 levels were increased when compared to controls. However, a direct comparison of this study to ours is not appropriate for two reasons. First, the pathophysiology of childhood asthma may not necessarily resemble that found in adult asthmatics. Age-related changes in eosinophil function have been documented recently. A comparison of young adults (20–38 yr) and older adults (57–75 yr) found eosinophil degranulation owing to IL-5 stimulation to be decreased in the older group, although percentage sputum eosinophils were similar (21). Second, the eosinophil percentage level in Xu et al.'s SA group was much higher than in ours (20.3% vs. 4.6%). IL-5's role may differ depending on the severity of asthma. Another study found their mild asthma group exhibited no change in IL-5 levels before and after corticosteroid treatment in contrast to a significant decrease observed in the severe asthma group (22). These findings raise the possibility that airway eosinophils are less dependent on IL-5, at least in children with SA. In contrast, CC

chemokines have been found to be highly potent chemoattractants for eosinophil recruitment (2). It is thought that damage to the bronchial mucosa in asthma causes secretion of eotaxin by airway epithelial cells (23). Therefore, we suggest that the increased eotaxin levels found in sputa are a direct reflection of their concentrations in airway mucous secretions. It has been reported that high eotaxin levels can induce eosinophil migration *in vitro* and may be important in the pathogenesis of airway eosinophilia found in asthmatics. In our study, there was a significant correlation between eotaxin levels and percentage eosinophils in patients with asthma. These results concur with Scheicher et al.'s findings (24). Fujisawa et al. (25) observed that CC chemokines, including eotaxin, induced eosinophil degranulation (EDN release). Furthermore, positive correlations between eotaxin and the eosinophil degranulation product ECP have been found in other studies (26). These results could explain the good correlation of eotaxin with EDN and ECP levels in our study. Our results in conjunction with those from animal and human studies suggest that the CC chemokine eotaxin may play an important role in eosinophil degranulation, at least in our pediatric asthma subjects.

The effects of ICS on sputum fluid-phase mediator levels are controversial. In our study, a total of 18 out of the 30 patients with asthma were taking ICS. There has been some evidence of the effects of topical corticosteroids on IL-5 levels in asthmatics (27). Therefore, one could argue that the lack of IL-5 increase and the lack of correlation between IL-5 levels and sputum eosinophil indices in our study were attributable to the effects of corticosteroids. However, in a study to examine the effect of ICS on sputum IL-5 in children with asthma, there were no significant differences in IL-5 levels before and after inhalation of corticosteroids (28). This was true in our study, as ICS did not significantly affect IL-5 levels. It has also been suggested that ICS may (26) or may not (29) affect eotaxin levels. Our study showed significant differences in eotaxin levels between the steroid and steroid-naïve subgroups of stable asthmatics. When comparing eosinophil indices (EDN, ECP levels, and percentage eosinophils) with IL-5 and eotaxin in the steroid subgroup, IL-5 did not correlate, whereas eotaxin did. Therefore, regardless of steroid use, eotaxin correlated well with eosinophil indices.

We also found no significant differences in percentage eosinophils, EDN, or ECP levels between the steroid and steroid-naïve subgroups. The level of ECP in induced sputum is considered a reliable marker of asthmatic airway inflammation (12). Our findings suggest another eosinophil degranulation product, EDN, could also be a reliable marker in asthmatics, regardless of steroid treatment.

Although patients with allergic asthma and AR in our study exhibited a similar pattern of eosinophilic inflammation in blood and sputum, only the asthmatic group presented with significant bronchial hyper-responsiveness (BHR). This suggests that factors in addition to eosinophilic inflammation in bronchial mucosa may determine whether an allergic patient develops asthma or not (30).

One potential limitation of this study is we were unable to exclude the possibility that the increased EDN and ECP levels found in sputum may partially be attributable to mechanical degranulation of eosinophils during sputum processing. However, we attempted to minimize the release of granule proteins during processing by: (i) minimizing the time between sputum expectoration and processing and (ii) maintaining the samples at 4°C for the entire time, as per standard protocol.

In conclusion, we propose that the CC chemokine eotaxin plays a much more significant role than the Th2 cytokine IL-5 in eosinophil degranulation. Consequently, our results do not support a strong role for IL-5 in children with SA. Indeed, mechanisms of eosinophil

degranulation different from those in allergen challenge models or acute asthma exacerbation may orchestrate the pathology found in these patients.

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Abbreviations

AA	atopic asthma
AC	atopic control
DTT	dithiothreitol
ECP	eosinophil-cationic protein
EDN	eosinophil- derived neurotoxin
FEV₁	forced expiratory volume in 1 s
GM-CSF	granulocyte macrophage colony stimulating factor
IL	interleukin
MCP-3	monocyte chemoattractant protein-3
NC	non-atopic normal control
RANTES	regulated on activation, normal T-cell expressed and secreted

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Fig. 1.

Comparisons of Th2 cytokine IL-5 (a) and CC chemokine eotaxin (b) levels in the three groups. IL-5 levels were not significantly different among the three groups, while eotaxin levels were significantly elevated in children with stable asthma. Mean values are represented as horizontal bars. The dotted line in (a) represents the sensitivity level of the assay.

Fig. 2.

Correlations of Th2 cytokine IL-5 with sputum eosinophil indices, percentage eosinophils (a, b), eosinophil-derived neurotoxin (EDN) (c, d), and eosinophil-cationic protein (ECP) (e, f) in children with stable asthma. Sputum IL-5 levels showed no correlations, while sputum eotaxin levels correlated significantly with sputum percentage eosinophils, EDN, and ECP levels. A p value of <0.01 was considered statistically significant.

Table 1

Baseline characteristics and eosinophil indices of sputa

	Stable asthma (n = 30)	Allergic rhinitis (n = 21)	Healthy control (n = 22)
Age (yr)	9.2 ± 2.4	9.0 ± 3.0	10.4 ± 3.7
Sex (M:F)	17:13	12:9	10:12
Atopy, n	30	21	0
Blood eosinophils (per mm ³)	504.1 ± 384.6*	654.6 ± 476.5*	188.4 ± 134.6
FEV ₁ , % predicted	88.2 ± 16.5	91.7 ± 19.3	92 ± 10.3
On ICS**/OCS†	18/0	0/0	0/0
Sputum eosinophils, %	4.6 ± 6.7‡	1.5 ± 2.1	(0.8 ± 1.2)
Sputum EDN, ng/ml	398.5 ± 213.6§¶	236.8 ± 154.1§	49.2 ± 27.5
Sputum ECP, ng/ml	245.9 ± 202.5§	131.0 ± 140*	31.5 ± 42.0

Data are expressed as mean ± s.d.

*
p < 0.001 compared with healthy control.†
Receiving OCS.‡
p < 0.01 compared with healthy control.§
p < 0.0001 compared with healthy control.¶
p < 0.01 compared with allergic rhinitis.**
Receiving ICS.FEV₁, forced expiratory volume in 1 s; ICS, inhaled corticosteroids; OCS, oral corticosteroids; EDN, eosinophil-derived neurotoxin; ECP, eosinophil-cationic protein.