

Urine leukotriene E₄ and eosinophil cationic protein in nasopharyngeal aspiration from young wheezy children

Oh J-W, Shin S-A, Lee H-B. Urine leukotriene E₄ and eosinophil cationic protein in nasopharyngeal aspiration from young wheezy children. *Pediatr Allergy Immunol* 2005; 16: 416–421. © 2005 Blackwell Munksgaard

Respiratory syncytial virus (RSV) infection is a risk factor for the development of asthma. It is very hard to distinguish bronchiolitis with respiratory virus infection from allergic asthma at first wheezing attack in early childhood. To distinguish wheezing children with RSV bronchiolitis from asthmatic children, we measured leukotriene E₄(LTE₄) in urine and ECP in nasopharyngeal aspiration (NPA) at first day of admission with wheezing attack. Thirty-two non-atopic children younger than the age of 3 yr with RSV induced bronchiolitis, 35 atopic asthmatic children with/without respiratory viral infection, and 23 children who exhibited no evidence of atopy, asthma, or virus infections as controls were selected in this study. We measured urinary LTE₄ and ECP level in NPA from subjects. Urinary LTE₄ concentrations in children with asthma were significantly higher than urinary LTE₄ in bronchiolitis and in controls (240.8 ± 129.8 vs. 162.8 ± 73.9 vs. 85.1 ± 31.6 pg/ml). Children with RSV infection demonstrated higher urinary LTE₄ levels compared to children without RSV infection among asthmatic children. ECP in NPA was significantly correlated with urinary LTE₄ ($r = 0.57$, $p < 0.01$) in children entered this study who had detectable levels for both LTE₄ and ECP. In summary, Urinary LTE₄ concentrations may be suggested to useful mediators for differential diagnosis of wheezy diseases in early childhood. RSV infection also is associated with synergizing LT biosynthesis and this study demonstrated ECP in NPA was significantly correlated with urinary LTE₄ and may suggest that cysteinyl leukotriene initiate the production of ECP in early childhood, which could contribute to the development of wheeze.

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Key words: children; asthma; leukotriene; eosinophil; respiratory syncytial virus; wheeze

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Accepted 4 May 2005

Recurrent wheezing in early childhood may have a multitude of causes, such as respiratory virus infections and airway inflammation associated with the atopic trait (1). A number of studies suggest that there may be synergistic immunological interactions after viral respiratory infections and allergic airway sensitization (2). Respiratory syncytial virus (RSV) is the most common respiratory pathogen in early childhood and RSV infection is an important risk factor for the development of asthma (3). However, the mechanisms underlying such interactions have not been well defined, although many potential

mechanisms have been suggested for the exacerbation of airway hyper-reactivity by viral infections. It is very hard to distinguish bronchiolitis with respiratory virus infection from allergic asthma at first wheezing attack in early childhood.

The measurement of urine leukotriene E₄ (LTE₄) is a useful non-invasive method to assess changes in the rate of total body cysteinyl leukotrienes (cysLTs) production. In recent study, urinary LTE₄ concentration is correlated with the degree of airflow obstruction and airway inflammation in children with atopic asthma (4).

Recently cysLTs are of particular interest in RSV induced respiratory diseases because providing evidences that production of virus specific IgE antibody and release of cysLTs occurs in the airways of infants with virus-induced wheezing (5, 6).

CysLTs have been reported to promote recruitment and inhibit apoptosis of eosinophils (7). In addition, LTD₄ was also shown to augment the growth of eosinophilic precursors from bone marrow or peripheral blood stimulated by either interleukin-5 or granulocyte macrophage colony-stimulating factor (8, 9). A number of studies have supported that ECP in induced sputum or nasopharyngeal secretion may be a useful marker in symptomatic children with asthma. And LTE₄ in urine and ECP in nasopharyngeal aspiration (NPA) are very associated with allergic diseases as well as respiratory virus infection.

Therefore the authors postulated that it might be possible to distinguish between wheezing children with RSV bronchiolitis and asthmatic children by measuring LTE₄ in urine and ECP in NPA at first day of admission with wheezing attack.

Methods

Subjects

Non-atopic children with RSV induced bronchiolitis. Thirty-two children were selected from patients younger than the age of 3 yr who had been diagnosed as having a RSV infection. Bronchiolitis was defined as acute respiratory illness with wheezing, tachypnea or dyspnea with intercostal recession, prolonged expiration, and wheezing on auscultation. Their chest X-rays were consistent with a diagnosis of bronchiolitis. The patients had primary viral infections with negative personal and family histories of allergy, and the total serum IgE levels in the subjects were within the age-matched normal range (Table 1).

Table 1. Characteristics of subjects

	Atopic asthma	RSV induced bronchiolitis	The controls
Age (months)	27.3 ± 6.4	13.8 ± 7.8	16.5 ± 9.7
Male:Female	22:13	19:13	13:10
FAS	9.6 ± 8.3	1.5 ± 2.4	0
Total Eosinophil (/mm ³)	273.2 ± 183.2*	46.2 ± 27.5	58.4 ± 61.3
Total IgE (IU/mL)	83.7 ± 97.5*	21.5 ± 20.8	19.3 ± 20.4

FAS, family allergy score system.

*p < 0.05.

Atopic asthmatic children with/without respiratory viral infection. Thirty-five asthmatic children with an age of 27.3 ± 6.4 months (mean ± s.d.) were entered in this study (Table 1). Asthma was defined as to have made three or more visits to the clinic/hospital for wheezing attacks during last 2 yr. When children were admitted at hospital, 23 children were classified as having mild asthma and twelve children had moderate to severe asthma by GINA guideline (10). Fifteen children had RSV infection among asthmatic children group.

Atopy was defined as having at least one positive skin prick test to common allergens (egg, milk, soybean, hazelnut, peanut, cod, crab, shellfish mix, cat fur, dog hair, grass mix, tree mix, *Dermatophagoides farinae*, *D. pteronyssinus*, *Cladosporium herbarum*, *Alternaria areata*; ALK, Copenhagen, Denmark), and a positive response to histamine and having positive personal or family histories of allergy and age-matched, high IgE level in serum. Candidate subjects who had used topical or systemic corticosteroids within 6 wk prior to the day of study were excluded. Family allergy score (FAS) system was used for assessment of family allergy history (Table 2).

The controls. Twenty-three non-atopic healthy children aged 3–36 months who exhibited no evidence of atopy, asthma, or virus infections were recruited as the controls.

The study was done only after informed consent was obtained and approval of the ethics committee of Hanyang University Hospital was given.

Collection and storage of samples

Urine was obtained from each child in the laboratory prior to the performance of the lung function test. The samples were immediately centrifuged to remove cellular debris at 10,000 × g for 10 min, the supernatant was then removed, coded and stored in aliquots of 5 ml at –70°C until analysis.

Table 2. Family allergy score system

	Obvious*	Score	Probable**	Score
Father	(+, –)	3	(+, –)	2
Mother	(+, –)	3	(+, –)	2
Siblings	(+, –)	3	(+, –)	2
Grandparents	(+, –)	2	(+, –)	1
Uncle or aunt	(+, –)	2	(+, –)	1

*Have ever diagnosed allergic diseases by medical doctors.

**Mean suspicious allergic diseases, but have never been diagnosed allergic diseases.

Analysis of LTE_4

LTE_4 in urine was measured ACETM Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, MI, USA) a sensitive and specific enzyme immunoassay. This assay is based on the competition between free LTE_4 and LTE_4 tracer for a limited number of LTE_4 -specific rabbit anti-serum binding sites. All measurements were done in duplicate and the mean value was calculated. The detection limit in the assay was < 8 pg/ml.

Collection of NPA and identification of respiratory virus in NPA

Samples of nasopharyngeal secretions were obtained by aspiration from the nasopharynx through catheters after instilling 5 ml of sterile physiologic saline solution into mucus traps at the time of hospitalization. Each specimen was mixed well and centrifuged at 1500 g for 10 min. Supernatants were kept at -20°C until analysis for ECP-ELISA. Meanwhile, the precipitate was subjected to the indirect immunofluorescent antibody assay for the qualitative identifications of respiratory viruses (Chemicon International, Temecula, CA, USA).

ECP analysis

ECP was analyzed by a commercially available monoclonal antibody-based fluorometric assay (Pharmacia CAP FEIA, Pharmacia Diagnostics, Uppsala, Sweden). The lower detection limit was < 0.5 $\mu\text{g/l}$. NPAs from each of the children were analyzed in duplicate. The results were expressed in $\mu\text{g/l}$.

Measurement of total serum IgE level and blood eosinophil count

An amount of 10 ml of whole bloods was drawn from each subject and took 5 ml of them for eosinophil count and the other was centrifuged and took the serum for IgE. Total serum IgE was measured by radioimmunosorbent assay (Kabi-Pharmacia, Uppsala, Sweden), with a lower detection limit less than 1.5 IU/ml.

Statistical analysis

Statistical analyses were performed with SPSS 7.5 for Windows. Statistical comparisons of LTE_4 level in urine and ECP in NPA among the groups were performed using non-parametric test (Kruskal-Wallis test) and Mann-Whitney was applied for the comparison of groups. Values of $p < 0.05$ were considered statistically significant.

Correlation analysis between LTE_4 and ECP levels was performed by, using the *Spearman* rank correlation test.

Results

Ninety children were enrolled over a period of 3 yr. Total eosinophil counts in peripheral blood were significantly different between atopic children with asthma and non-atopic children with bronchiolitis (273.2 ± 183.2 vs. 46.2 ± 27.5). Serum IgE in asthmatic children was also higher than children with bronchiolitis, however, comparison of mean value is not meaningless in these ages because comparison of age matched value needs under 2-year old (83.7 ± 97.5 vs. 21.5 ± 20.8) as shown in Table 1.

Urine LTE_4 and serum ECP level between children with asthma and bronchiolitis

Urinary LTE_4 concentrations were significantly higher in children with asthma than in bronchiolitis and in controls (240.8 ± 129.8 vs. 162.8 ± 73.9 vs. 85.1 ± 31.6 pg/ml; $p < 0.05$; Fig. 1). Among asthmatic children group, children with RSV infection demonstrated higher urinary LTE_4 levels compared to children without RSV infection as shown Fig. 2. In addition, comparison between the group of mild and moderate to severe asthmatics revealed no significant difference in urinary LTE_4 concentration. Concentrations of ECP in asthmatic children with or without RSV infection (235.9 ± 114.5 ng/ml) were significantly higher than the mean concentration of

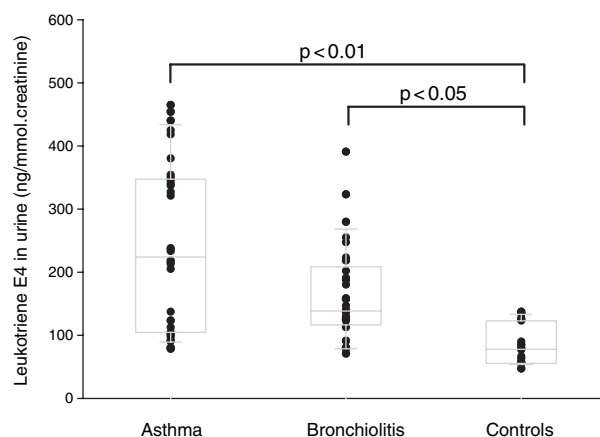


Fig. 1. The comparison of urinary LTE_4 levels among asthmatic children with or without RSV infection, children with RSV induced bronchiolitis, and the controls. Urinary LTE_4 levels were significantly higher in asthmatic children than in children with RSV induced bronchiolitis ($p < 0.05$), and in the controls ($p < 0.01$).

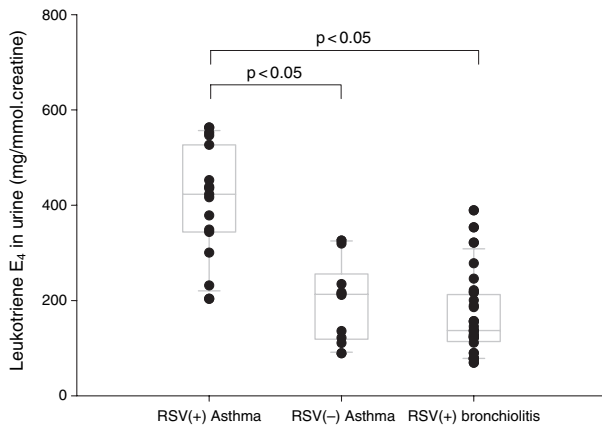


Fig. 2. The comparison of urinary LTE₄ levels among asthmatic children with RSV infection, asthmatic children without RSV infection, and RSV induced bronchiolitis. Urinary LTE₄ levels of asthmatic children with RSV infection were significantly higher than those of asthmatic children without RSV infection and children with RSV induced bronchiolitis ($p < 0.05$).

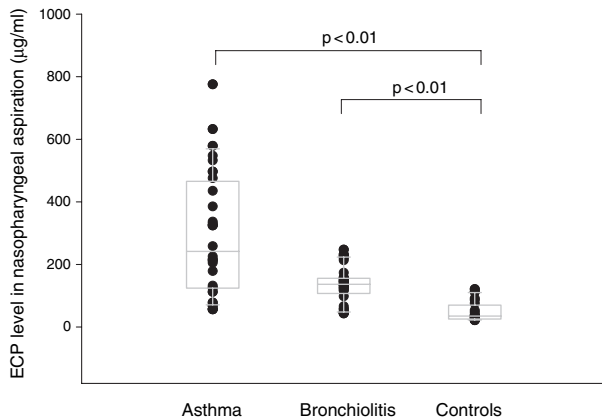


Fig. 3. The comparison of ECP levels in NPA among asthmatic children with or without RSV infection, children with RSV induced bronchiolitis, and the controls. ECP levels in NPA of asthmatic children and children with RSV induced bronchiolitis both were significantly higher than the controls ($p < 0.01$).

ECP in both groups of children with RSV bronchiolitis (133.9 ± 55.3 ng/ml, $p < 0.01$) and controls (48.7 ± 31.1 ng/ml; $p < 0.01$), whereas children with RSV induced bronchiolitis had levels of ECP in NPAs significantly higher than controls (Fig. 3).

Correlation between Urine LTE₄ and serum ECP levels

ECP in NPA was significantly correlated with urinary LTE₄ ($r = 0.57$, $p < 0.01$) in children entered this study who had detectable levels for both LTE₄ and ECP (Fig. 4). There were no

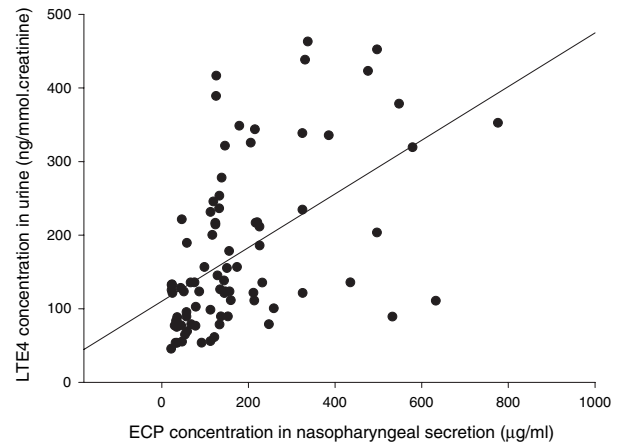


Fig. 4. Correlation between Urine LTE₄ and serum ECP levels. ECP in NPA was significantly correlated with urinary LTE₄ ($r = 0.57$, $p < 0.01$) in children entered this study who had detectable levels for both LTE₄ and ECP.

associations with family history of asthma or allergic disease (data not shown).

Discussion

In present study, urinary LTE₄ level from asthmatic children is significantly higher than those from children with acute RSV induced bronchiolitis. However, there was not shown significant difference between urinary LTE₄ level from asthmatic children without RSV infection and from children with acute RSV induced bronchiolitis when divided the asthmatic children into asthmatic children with RSV infection and without RSV infection.

The present results support the previous study that increased cysLT production during acute preschool viral wheeze is associated with high serum IgE (11). Therefore, asthmatic attack and RSV infection both can stimulate the biosynthesis of cysLTs, whereas there was not significantly different between concentrations of cysLTs from both these conditions in early childhood. But RSV infection in the atopic children with asthma may revoke synergic effect to increase biosynthesis of cysLTs compared with RSV infection in non-atopic children.

Approximately 7% of a total inhaled dose of LTC₄ appears in the urine as LTE₄, indicating that measurement of urinary LTE₄ provides a useful indication of *in vivo* cysLT production in the lung (12). There was well known that the cysLTs are potent mediators of airway narrowing, edema, smooth muscle proliferation, and increased mucus production derived from the lipoxygenation of arachidonic acid during asthmatic attack and the LTs mediate changes in the

airway consistent with the pathobiology of asthma through a number of studies (13, 14).

Some studies demonstrated that RSV infection in bronchial epithelial cells induces expression of the 5-lipoxygenase (5-LO) gene, which plays a leading role in the synthesis of LTs, and that RSV infection enhance both the generation of and the responses to cysLTs (15). A number of study reported that increased levels of LTC₄ were measured in nasopharyngeal secretions of children with RSV-induced bronchiolitis as compared to children with upper respiratory tract infection due to RSV (16). Based on these characteristics, release of LTs into the airway during respiratory virus infection offers a possible explanation for the wheezing observed in bronchiolitis.

ECP is one of the cytotoxic proteins released from the granules of activated eosinophil, which has been considered as having an important role in the pathogenesis of allergic diseases (17).

The present data demonstrated asthmatic children with or without RSV infection were shown higher ECP levels in NPA compared with children with RSV induced bronchiolitis, whereas RSV induced bronchiolitis is higher than the controls.

Elevated levels of ECP have also been reported in nasopharyngeal secretions and serum of children with RSV induced bronchiolitis compared with those with RSV negative bronchiolitis, whereas the total eosinophil counts were significantly decreased in all patients with RSV induced bronchiolitis, whereas eosinophils are differently found in serum from atopic children asthma and infants with RSV induced bronchiolitis (18, 19). Considering these facts, eosinophil count may not be proportion to ECP level in serum or nasopharyngeal secretion from children with bronchiolitis. Some researchers speculated that ECP originate from neutrophils as well as eosinophils, because neutrophils contain mRNA transcripts for ECP and eosinophil-derived neurotoxin (20, 21). Furthermore, the other studies suggested that mast cells and neutrophils might be the stimulators of eosinophils to produce ECP (22).

In addition, the association between LTE₄ and ECP is consistent with the function of eosinophil to release biologically active lipids, which the oxidative metabolism of arachidonic acid by the 5-LO pathways in eosinophils leads to the intracellular degranulation and subsequent release of cysLTs (23). The elaboration of eicosanoid by eosinophils differs from the predominant formation of LTB₄ from neutrophils, that is, eosinophils possess a specific LTC₄ synthase, which conjugates glutathione to the intermediate

LTA₄ to produce LTC₄ (24). It has been suggested that eosinophil plays an important role in host defense against RNA viruses by *in vitro* and *in vivo* (25, 26). These suggestions might contribute a cysLTs synthesis and eosinophil migration for defense against virus could become established in the airway during RSV induced bronchiolitis.

This association between urinary LTE₄ and ECP in NPA might be explained by the potent chemoattractant properties of cysLTs for eosinophils. This significant independent positive association between them implies that the eosinophil was the most likely source of LTC₄ in the children we studied.

Taken together, RSV infection is associated with enhanced LT synthesis in the respiratory tract and recruitment of inflammatory cells such as eosinophil into airways and may synergize LT biosynthesis in atopic children. In addition, RSV infection as well as allergens may initiate the production of ECP in early childhood, which could contribute to the development of wheeze, although it is unclear as to whether ECP comes from eosinophils or whether they originate from other cells.

Acknowledgment

This study was supported financially by a clinical research fund of Hanyang University (HY-2002), Seoul, Korea.

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