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Original article

Study of urinary leukotriene E4 in atopic dermatitis: relation to disease severity.

Background: *Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease prevalent in patients with a personal or family history of atopy. Cysteinyl leukotrienes (LTs) are inflammatory mediators which play a role in the pathogenesis of atopic diseases. Urinary leukotriene E4 (LTE4) has been used as an index of the whole body cysteinyl LTs production.*

Objective: *This study was meant to evaluate the importance of LTs in atopic dermatitis (AD) and to study the correlation of urinary LTE4 with disease severity and some commonly altered parameters in AD.*

Methods: *The study included 30 children and adolescents diagnosed to have atopic dermatitis. Ten age and sex matched healthy children and adolescents were enrolled for comparison. They were subjected to clinical evaluation and measurement of urinary LTE4, absolute eosinophilic count, serum IgE and IL-4 and IL-5 in peripheral blood mononuclear cell culture (PBMC) supernatant. The patients were categorized into mild (n=5), moderate (n=16) and severe (n=9) AD subgroups.*

Results: *The study revealed a significant increase in absolute eosinophilic count, urinary LTE4, serum IgE and IL-4 and IL-5 in PBMC culture supernatant in the patients as compared to controls. Moreover, urinary LTE4 levels were significantly increased in moderate and severe cases of AD as compared to the control group, whereas mild cases had levels that were comparable to the controls. Urinary LTE4 levels were higher in severe ($p<0.01$) and moderate cases ($p<0.05$) when compared to mild cases. Significant positive correlations could be elicited between urinary LTE4 and PBMC IL-4, disease severity scale, absolute eosinophilic count and serum total IgE. However, urinary LTE4 could not be correlated statistically with PBMC IL-5.*

Conclusion: ***E****levation in urinary LTE4 excretion in AD patients was demonstrated reflecting increased production of cysteinyl LTs. Urinary LTE4 was correlated to clinical and laboratory markers of severity suggesting that it could be an easy, non invasive and objective prognostic test in AD. Trials of 5-lipoxygenase inhibitors and LT receptor antagonists as additional lines of therapy in AD could thus be suggested.*

Key words: atopic dermatitis, urinary LTE4, IgE, IL-4, IL-5, eosinophilic count.

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INTRODUCTION

There is increasing evidence that the frequency of atopic diseases including atopic dermatitis has increased over the last few decades with prevalence rates ranging between 10-20%[1]. Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease seen in patients with a personal or family history of atopy. Understanding the mechanisms that underlie AD has important clinical implications for the development of new management protocols for this illness[2].

IgE-mediated mechanisms play a mutlifactorial role in the pathogenesis of AD. Serum IgE levels are elevated in 80-85% of AD patients and is considered an important hallmark of the disease[3]. Eosinophilia is another important finding in the peripheral blood of the allergic type[4] and eosinophils play an effector role in AD as they

migrate from the blood into lesional skin and release cytotoxic mediators[4]. TH2 cytokines as IL-4[5,6] and IL-5[7] are usually elevated in peripheral blood of AD patients. Their elevation contributes to the elevated peripheral blood IgE level [8,9,10].

Leukotrienes (LTs) are phospholipids derived from arachidonic acid in a pathway involving phospholipase 5-lipoxygenase and 5-lipoxygenase-activating proteins. The cysteinyl LTs (LTC4, LTD4 and LTE4) are inflammatory mediators which are suggested to play an important role in the pathogenesis of AD although their exact cellular source in patients with AD is not specified (possibly eosinophils, basophils, and mast cells)[11]. LTE4 is a stable urinary metabolite of LTC4 and LTD4, and is a useful index for the body production of these cysteinyl leukotrienes[12].

With this as background we thought to investigate the role of urinary LTE4 in AD and anticipate its possible correlation with clinical severity and other laboratory parameters as absolute eosinophilic count, serum IgE and IL-4 and IL-5 levels in PBMC culture supernatant.

METHODS

This study was conducted on 30 children and adolescents diagnosed to have atopic dermatitis according to the UK refinement of Hanifin and Rajka's diagnostic criteria for AD[13]. They were recruited consecutively from the outpatient clinics of the Pediatric and Dermatology Departments of Ain Shams University Hospitals. They were 14 males and 16 females and their ages ranged between 3.7 and 11.2 years with mean age of 7.4 ± 2.6 years. Atopic dermatitis was graded into mild, moderate and severe according to the grading system of Rajka and Langeland[14]. Accordingly, 5 patients were considered to have mild , 16 had moderate and 9 had severe AD.

Inclusion criteria:

Patients stopped systemic and topical treatments before the study for at least one month and 2 weeks respectively. Antihistaminics were also stopped one week before the study and only topical emolliants were used. Patients who concomitantly had any chronic systemic illness or dermatologic diseases apart from atopic dermatitis were excluded from the study.

Controls:

Ten apparently healthy age and sex matched children and adolescents were included as controls. They were 5 males and 5 females and their ages ranged between 3.8 and 11.7 years with mean age of 6.9 ± 2.8 years. They were enrolled after exclusion of a personal or family history of atopy or chronic systemic or dermatologic diseases.

An informed consent was obtained from parents of each subject.

Measurements:

Patients were subjected to the following:

1. History taking laying stress on personal and/ or family history of atopy, distribution of skin lesions and effect of allergens and emotional stress on skin lesions. History of recurrent conjunctivitis and skin infections was also sought for.
2. Clinical examination to exclude systemic illnesses, stunted growth and nutritional disorders.
3. Skin examination for xerosis, icthyosis, palmar hyperlinearity, cutaneous infection, Dennie Morgan infraorbital folds, orbital darkening, mask of atopic dermatitis, hyperpigmentation, scaling, lichenification and white dermographism as well as assessment of severity according to the grading system of Rajka and Langeland[14].
4. The following laboratory investigations were performed for patients and controls:

I-Complete blood count using Coulter cell counter T660 (Coulter corporation, Florida, USA) with examination of Leishman stained peripheral blood (PB) smears for estimation of the absolute eosinophilic count.

II- Measurement of urinary LTE4 by ELISA (Neogen, Lexington, KY, USA). Urine samples were collected after 6 hours without voiding, kept at -70°C till the time of measurement. After extraction, 1ml of urine was acidified to pH 3.5 with 1 NHCL. Preparation of standards was done followed by addition of lµl of conjugate to 50 µl total volume of EIA buffer then, 50 µl of standards and samples were added to wells in duplicate. 50 µl of the diluted enzyme conjugates were added to each well and mixed by gentle shaking, covered and incubated for 1 h at room temperature. 20 ml of wash buffer was diluted in 180 ml of deionized water. After incubation, contents were dumped out then tapped out thoroughly on a clean towel and washed three times. Addition of 150 µl substrate to each well followed by 30 minutes incubation were done. Gentle shaking and reading the plate in microplate reader was done[15].

III-Creatinine in the urine was determined using a creatinine test kit (Wako Pure Chemical Industries, UK). The results displayed are corrected values.

IV- Quantitative determination of serum IgE concentrations: 2 ml of venous blood were collected in a tube, left to clot and centrifuged at 1500 rpm to separate the serum. Estimation of serum levels of IgE were done using the ELISA technique. (Pathozme IgE, Omega Diagnostic Limited, UK)[16].

V- Measurement of levels of IL-4 and IL-5 in PBMC culture supernates: Five ml of venous blood were drawn aseptically into a disposable syringe containing heparin- free preservative to give a final concentration of 10 IU/ml of blood. In sterile centrifuge tubes, 5 ml blood were carefully layered over 3 ml sterile ficoll hypaque mixtured and centrifuged for 15 minutes, followed by collection of mononuclear cells being washed twice in sterile complete RPMI 1640 (Gibco, Grand Island, NY, USA). It contained L-Glutamine, penicillin, streptomycin and N-2 Hydroxyethyl piperazine and was centrifuged for 10 minutes. The final cell pellet was gently tapped and re-suspended in a known volume of complete RPMI 1640 medium to which 200 µl of PHA was added as mitogen and incubated for 72 h at 37°C in 5% C02. The suspension was taken in a test tube, centrifuged and kept at 20°C till time of assay. Estimation of culture supernatant IL-4 and IL-5[17] was done by sandwich enzyme immunoassay technique (R & D Systems, Inc., Mckinley Place NE, Minneapolis, USA).

Statistical analysis:

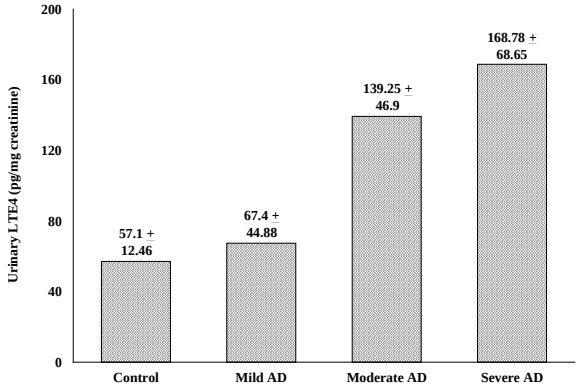
The data were analyzed statistically using SPSS statistical package version 11 using student t-test for independent samples as well as ANOVA (analysis of variance), post hoc and Pearson's correlation coefficient (r) tests. p values < 0.05 were considered significant.

RESULTS

The AD patients were found to have significantly higher levels of absolute eosinophilic count, urinary LTE4, serum IgE and IL-4 in PBMC culture supernatant fluid ($p<0.001$) as well as PBMC IL-5 ($p<0.05$) compared to the controls (Table 1).

Table 2 shows that urinary LTE4 levels were significantly higher in moderate as well as severe cases of AD than controls, whereas mild cases were comparable to controls in urinary LTE4 levels. Urinary LTE4 mean level was also higher in severe ($p<0.001$) and moderate cases ($p<0.05$) as compared to mild cases. However, no significant difference could be elicited between moderate and severe cases (Fig.1). The absolute eosinophilic count was significantly elevated in moderate ($p<0.05$) and severe ($p<0.01$) cases of AD as compared to controls. Again, it was significantly higher in severe compared to mild and moderate cases, while no statistically significant difference was found between mild cases and controls or between mild and moderate cases of AD. The serum total IgE levels were significantly higher in moderate ($p<0.05$) and severe ($p<0.01$) cases of AD than the control group and were significantly increased in severe as compared to moderate and mild cases of AD. On the other hand, no statistically significant difference was detected between mild and moderate cases or between mild cases and controls. A highly significant elevation was found in the mean level of PBMC-IL-4 in moderate and severe cases of AD as compared to controls and in severe AD patients as compared to mild and moderate cases. It was also significantly increased in moderate as compared to mild cases of AD. On the contrary, its level in mild AD cases was comparable to that of controls. The mean level of PBMC-IL-5 was significantly higher in severe cases of AD as compared to controls and to mild and moderate cases of AD. However, other groups were comparable in their PBMC IL-5 mean levels.

Urinary LTE4 could be positively correlated to disease severity scores ($r=0.69$, $p<0.001$), absolute eosinophilic count ($r=0.41$, $p< 0.001$), serum total IgE (*figure* 2) and PBMC IL-4 (figure 3). On the other hand, urinary LTE4 did not correlate significantly to PBMC IL-5 ($r= 0.3$, $p> 0.05$).



F value by ANOVA test = 12.009 ($p<0.01$)

AD: atopic dermatitis; LTE4: leukotriene E4.

Figure 1: Variation of urinary LTE4 of enrolled subjects according to AD severity in comparison to controls.

Table 1: Laboratory parameters of the studied sample.

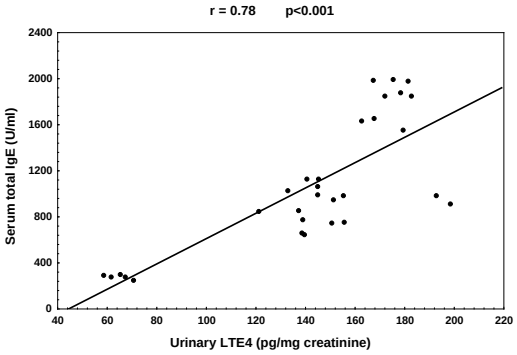
Laboratory parameters	AD Patients (n=30)	Controls (n=10)	t value	p value
Urinary LTE4 (pg/mg creatinine)	136.13± 62.18	57.10 ± 12.46	6.58	<0.001
Absolute eosinophilic count (10^9/L)	0.64 ± 0.49	0.18 ± 0.13	4.71	<0.001
Serum total IgE (u/ml)	1019.27± 937.70	63.41 ±29.67	5.04	<0.001
PBMC-IL-4 (u/ml)	90.99 ± 68.80	0.53 ±0 .45	7.20	<0.001
PBMC-IL-5 (u/ml)	17.74 ± 14.03	0.39 ±0.34	4.45	<0.05

AD: atopic dermatitis; IgE: immunoglobulin E; IL-4: interleukin-4; IL-5: interleukin-5; PBMC: peripheral blood mononuclear cells.

Table 2: Variation of laboratory parameters of enrolled subjects according to AD severity in comparison to controls.

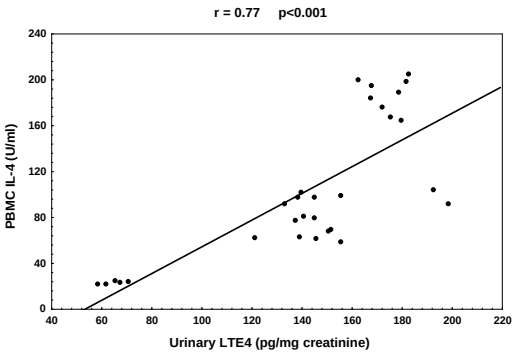
	Control group (A) (n=10)	Mild AD (B) (n=5)	Moderate AD (C) (n=16)	Severe AD (D) (n=9)	p values					
					B vs A	B vs C	B vs D	C vs A	C vs D	D vs A
Urinary LTE4 (pg/mg creatinine)	57.1 ± 12.46	67.4 ± 44.88	139.25 ± 46.9	168.78 ± 68.65	>0.05	<0.05	<0.01	<0.01	>0.05	<0.01
Blood eosinophils (10^9/L)	0.18 ± 0.13	0.28 ± 0.17	0.44 ± 0.26	1.2 ± 0.46	>0.05	>0.05	<0.01	<0.05	<0.01	<0.01
Serum IgE (U/ml)	63.41 ± 29.67	283.2 ± 214.1	778.81 ± 550.15	1855.67 ± 1430.16	>0.05	>0.05	<0.05	<0.05	<0.05	<0.01
PBMC IL-4 (U/ml)	0.53 ± 0.45	23.7 ± 12.3	63.73 ± 35.82	176.83 ± 45.52	>0.05	<0.05	<0.01	<0.01	<0.01	<0.01
PBMC IL-5 (U/ml)	0.34 ± 0.31	3.72 ± 2.31	6.09 ± 5.48	29.79 ± 11.45	>0.05	>0.05	<0.01	>0.05	<0.01	<0.01

AD: atopic dermatitis; IgE: immunoglobulin E; IL-4: interleukin-4; IL-5: interleukin-5; PBMC: peripheral blood mononuclear cells.



LTE4: leukotriene E4.

Figure 2: Positive correlation between urinary LTE4 and serum total IgE.



LTE4: leukotriene E4; PBMC: peripheral blood mononuclear cells.

Figure 3: Positive correlation between urinary LTE4 and PBMC IL-4.

DISCUSSION

The mean level of urinary LTE4 was significantly increased in atopic dermatitis patients compared to the control group (p<0.001). The same observation was reported by Fauler et al[18] and Hishinuma et al[2]. The enhanced urinary excretion of cysteinyl LTs can result from either enhanced synthesis or reduced hepatic elemination[18]. Since all our patients suffered no hepatic problems, so the increase in urinary LTE4 levels actually reflects an enhanced synthesis of these metabolites. This supports the role of cysteinyl LTs in the pathogenesis of AD which is probably mediated through increasing vascular permeability and dilating the skin blood vessels thus contributing to the inflammatory reaction in AD[19].

In contrast to our results, Sansom et al[20] found no significant difference in urinary LT levels in AD patients compared to the control group. This might be due to sample to sample variability within an individual which thus necessitates evaluation of multiple samples or longer urine collection rather than a single sample collected during a short

period[21]. This was considered in our study by taking urine sample after at least 6 hours without voiding.

Urinary LTE4 expression was significantly higher in moderate and severe cases of AD than mild ones. We also noticed a significantly positive correlation between LTE4 and disease severity. Despite the limited number of our series, the available data may suggest a beneficial value of LTE4 urinary level estimation as a simple, non invasive, objective prognostic test in AD. In the study of Miyoshi et al[22], urinary LTE4 levels were found significantly elevated in AD children who had severe nocturnal itching compared to those who had mild nocturnal itching and they suggested that increased production of leukotrienes might be relevant to nocturnal exacerbation of itching in children with atopic dermatitis. On the contrary, Yamamoto et al[23], reported that there was an increase in the urinary LTE4 in asthmatic patients than in normal subjects but there was no correlation between such increase in urinary LTE4 and the disease severity.

The current study revealed that the absolute eosinophilic count and the mean levels of serum IgE, PBMC-IL-4 and PBMC IL-5 were significantly increased in patients having AD than controls .These results are in agreement with many other studies as those of Tang et al[6] and Mochizuki et al[24]. We also observed that the aforementioned parameters were related significantly to grades of clinical severity (Table 2) which comes in accordance with other studies as those of Tang et al[6] and Mochizuki et al[24]. However, Kaminishi et al[25], stated that elevated levels of IL-4 in peripheral T-cells of patients with atopic dermatitis are not correlated with disease severity .

The above data confirm the importance of eosinophilia, increased serum IgE, IL-4 and to a lesser extent IL-5 in the pathogenesis of AD. Moreover, these data support the immunoregulatory abnormalities seen in patients with AD that lead to increased expression of Th2 cytokines as IL-4 and IL-5 which in turn stimulate the IgE synthesis by B cells and are chemotactic to eosinophils with a concomitant decrease of IFN-γ[26]. The number of eosinophils and the quantity of their products as cationic proteins and major basic proteins are found in previous studies to be increased in the peripheral blood of patients with AD and this increase was proved to be correlated with the clinical severity of the skin disease. The eosinophils infiltrate the affected skin in response to chemotactic substances as IL-4 and IL-5 generated by Th-2 cells. Moreover, in patients having AD, the eosinophils show enhanced response to these cytokines especially IL-4[27]. The previous studies proved that about 85% of patients with AD have increased amount of total IgE. Meanwhile, the mean levels of IgE were proved to increase in amounts with increasing severity and extent of dermatitis and patients with high levels were proved to have poorer prognosis[28,29]. The infiltrating lymphocytes in AD are capable of releasing a variety of interleukins that may promote mast cell proliferation (IL-3), cell surface expression of Fc ε receptors and IgE synthesis (IL-4) and eosinophil proliferation (IL-5). The presence of IL-4 and IL-5 in the AD lesions attract inflammatory cells such as eosinophils and macrophages to the area and prolong inflammation by preventing their apoptosis. IL-4 can also lead to steroid resistance by inducing abnormalities in glucocorticoid receptor binding affinity[30].

In the present study, a significant positive correlation between urinary LTE4 and the mean level of serum IgE was elicited. This result is in accordance with that of Hishinuma et al[2] .Moreover, we report for the first time the presence of a significant positive correlation between urinary LTE4 and both the absolute eosinophilic count and the PBMC IL-4. These findings suggest that increased levels of urinary LTE4 in AD might be due to increased total cellular response to stimulation with IgE which is in turn triggered by IL-4 and also suggest that eosinophils might be the source of increased cysteinyl LTs in AD and such increase in eosinophils could be due to stimulation by IL-4[24]. On the other hand, PBMC IL-5 showed no significant correlation with urinary LTE4.

In conclusion, this study demonstrates an increase in urinary LTE4 excretion in AD patients reflecting increased whole body production of cysteinyl LTs. Moreover, urinary LTE4 in AD correlated positively with disease severity making it an easy, non invasive, objective prognostic test in AD patients. Its correlation with eosinophil count, IgE and IL-4 suggests an IgE-dependent mechanism for its release and that eosinophils might be its possible cellular source. We thus recommend clinical trials on the effect of 5-lipoxygenase inhibitors and LT receptor antagonists as additional or alternative lines of therapy in AD.

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