SHORT REPORT

Enhanced peripheral leukocyte leukotriene production and bronchial hyperresponsiveness in asthmatics

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ABSTRACT: Leukotrienes (LTs) are pro-inflammatory mediators that contribute to the pathophysiological features of asthma. The relationship between the amounts of LTB₄ and LTC₄ produced by the leukocytes of asthmatic patients on the one hand and immunoglobulin E (IgE)-mediated allergy, asthma exacerbations and bronchial hyperresponsiveness was studied.

Leukocytes were obtained from peripheral blood drawn from 29 atopic and 27 nonatopic asthmatics during exacerbations and clinically controlled periods, as well as from 20 control individuals. The leukocytes were stimulated with calcium ionophore A23187 to induce LTB₄ and LTC₄ production. Allergy was assessed by means of specific serum IgE or by positive skin tests, whereas bronchial hyperresponsiveness was measured by methacholine challenge.

The leukocytes of the asthmatics generated significantly more LTB₄ ($p<0.05$) and LTC₄ ($p<0.01$) than those of controls. The leukocytes of patients with atopic asthma generated significantly more LTC₄ than those of patients with nonatopic asthma ($p<0.01$). Significantly more LTC₄ was produced by leukocytes obtained during exacerbations, than by those obtained during clinically controlled periods ($p<0.01$). In addition, there was a significant correlation between LTB₄ generation by leukocytes and the degree of bronchial hyperresponsiveness to methacholine ($r=-0.792$, $p<0.0001$).

These results suggest that leukotriene C₄ production by leukocytes is associated with immunoglobulin E-mediated allergy and asthma exacerbations, and further that generation of leukotriene B₄ is closely related to bronchial hyperresponsiveness in patients with asthma.

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Asthma is characterized by airway inflammation. Inflammatory cells such as lymphocytes, neutrophils and eosinophils, and a number of cytokines including leukotrienes (LTs) released from these cells, participate in the late asthmatic reaction. Among inflammatory cells, activated T-cells and eosinophils play an important role in the induction and persistence of the late asthmatic reaction [1]. Neutrophils have been shown to play a role in bronchial hyperresponsiveness during the late asthmatic reaction [2] and in severe persistent asthma [3, 4].

LTs are potent proinflammatory mediators that participate in the pathophysiological changes in the airways of patients with asthma [5]. Cysteinyl LTs (cys-LTs) induce bronchoconstrictor effects [6], increase mucus formation [7], and induce bronchial wall oedema [8]. LTB₄ stimulates neutrophil chemotaxis [9] and activates those cells, leading to the release of mediators and superoxides.

The present study assesses the relationship between the amounts of LTC₄ and LTB₄ generated by the peripheral leukocytes of patients with asthma and immunoglobulin E (IgE)-mediated allergy, asthma attacks and bronchial hyperresponsiveness.

Patients and methods

Patients

Fifty-six patients with asthma (36 female and 20 male; mean age 58 yrs) and 20 control individuals (11 female and 9 male; mean age 58 yrs), all of whom were lifetime non-smokers were examined. Asthma was diagnosed according to the criteria of the American Thoracic Society [10]. All patients with asthma were symptomatic, with episodic dyspnoea, wheezing and cough, and showed evidence of $\geq 15\%$ reversibility of forced expiratory volume in one second FEV₁ after inhalation of 200 μ g salbutamol. The normal controls had no history of wheeze or allergic diseases and their spirometric data were normal. Total and specific serum IgE were measured using the Pharmacia CAP System® (Pharmacia Diagnostics AB, Uppsala, Sweden). Diagnosis of atopy was based on specific serum IgE and/or a positive skin test for common inhalant allergens.

All patients had been inhaling β_2 -agonists as needed (table 1). Ten of the 29 atopic asthmatic patients had chronic severe asthma and had been on oral glucocorticoid therapy for >2 yrs (steroid-dependent intractable

Table 1. – Characteristics of controls and asthmatic patients

	Control	Atopic asthma		Nonatopic asthma	
		SDIA -	SDIA +	SDIA -	SDIA +
Participants n	20	19	10	16	11
Male/female n	9/11	7/12	4/6	6/10	4/7
Age yrs*	58±16	57±12	60±10	58±7	59±10
Serum IgE U·mL ⁻¹ †,¶	34±2	236±3**	261±3**	108±2**,#	140±3**
Specific IgE n	0	19	10	0	0
FVC % pred	108.5±7.7	100.5±18.0	95.0±15.9	96.4±11.0	90.9±20.3
FEV1 % pred	99.1±4.4	88.6±18.8	70.5±19.4*	88.4±13.8	75.5±20.2
FEV1/FVC %	82.0±6.3	68.1±9.8*	55.5±9.9*,§	66.1±7.3*	53.3±6.8*,§
Medication n					
Systemic corticosteroids	0	0	10	0	11
Oral β ₂ agonists	0	6	4	5	4
Theophylline	0	19	10	16	11
Inhaled corticosteroids	0	12	10	10	11
Sodium cromoglycate	0	3	2	3	2
LT generation ng·5 × 10 ⁶ leukocytes ⁻¹					
LTB ₄	43.2±24.4	83.2±31.4*	57.7±30.7	67.1±35.0*	55.0±35.4
LTC ₄	5.3±6.0	48.7±21.2**	8.4±5.5 ^{§§}	21.0±25.1**,###	6.0±5.8 ^{§§}

Data are presented as absolute values or mean±SD. †: geometric mean±SD. SDIA+: steroid-dependent intractable asthma (SDIA)-positive; SDIA-: SDIA-negative; IgE: immunoglobulin E; FVC: forced vital capacity; FEV1: forced expiratory volume in one second; LT: leukotriene. *, **: p<0.05, p<0.01 versus controls; #, ###: p<0.05, p<0.01 versus SDIA-atopic asthmatics; §, §§: p<0.05, p<0.01 versus SDIA-asthmatics.

asthma (SDIA)) [11] with a mean duration of the oral glucocorticoid regimen of 7.5 yrs and mean dose over the previous year of 7.0 mg·day⁻¹ prednisolone. Eleven of the 27 nonatopic asthmatic patients had SDIA (8.6 yrs; 7.3 mg·day⁻¹ prednisolone). The asthmatic patients who did not receive oral glucocorticoid therapy had been treated with other antiasthma drugs, including inhaled beclomethasone, but the dose was <400 µg·day⁻¹. None of the patients received LT modulators.

Patients were considered to be clinically controlled if they had been free of asthmatic attacks for ≥1 month, whereas the period <1 month after an exacerbation was still regarded as part of the exacerbation period. An asthma attack or escalation was defined as an abrupt and/or progressive worsening of shortness of breath, wheezing, chest tightness or a combination thereof, and/or a reduction in peak expiratory flow (PEF) to <70% of the personal best PEF obtained during clinically controlled period. Patients with an exacerbation associated with a bacterial respiratory infection were excluded from the protocol. This study was approved by the local research ethics committee, and all participants gave written informed consent.

Leukocyte stimulation and leukotriene measurement.

Inhaled β₂-agonists were withheld for 12 h, and other medications including systemic corticosteroids, inhaled beclomethasone, theophylline, oral β₂-agonists, sodium cromoglycate or antiallergic agents were withheld for 24 h prior to blood sampling during the clinically controlled period. Blood samples during the exacerbation period were taken before treatment for exacerbation (systemic corticosteroids). The amount of LTs, LTC₄ and LTB₄, generated by peripheral leukocytes was assessed as described previously [11].

Five millilitres 6% dextran (molecular weight ~200,000 kDa) (Nacalai Tesque, Inc., Kyoto, Japan) were added to 20 mL heparinized peripheral blood, and the resultant

mixture incubated for 1 h at room temperature. The density was adjusted to 5 × 10⁶ cells·mL⁻¹, and the cells were then stimulated with 1 µg calcium ionophore A23187. LTC₄ and LTB₄ were quantified by means of high-performance liquid chromatography, as described by LAM *et al.* [12].

Measurement of airway responsiveness

Bronchial responsiveness to methacholine was assessed in the asthmatics who did not receive oral glucocorticoid therapy during a clinically controlled period. Respiratory resistance (*R*_{rs}) during continuous inhalation of methacholine in stepwise increments was measured using a TCK 6100 Astograph (Chest Co., Tokyo, Japan) [13]. Methacholine was serially diluted two-fold with saline (in 10 dose steps from 25 mg·mL⁻¹ to 49 µg·mL⁻¹) and prepared for bronchial challenge, which proceeded according to the method of CHAI *et al.* [14]. The increase in *R*_{rs} after methacholine inhalation was measured using the forced oscillation technique. The minimum dose of methacholine *D*_{min} was used as an indicator of bronchial sensitivity. The *D*_{min} is the cumulative dose reached at the inflection point at which the reciprocal of *R*_{rs} (respiratory conductance (*G*_{rs})) decreases linearly. When the inflection point at which *G*_{rs} started to decrease was not clear, the point of intersection of the control value and the linear slope of *G*_{rs} was selected. The units for *D*_{min} are equivalent to 1 min of aerosol inhalation at 1 mg·mL⁻¹ during quiet tidal breathing. All medications were stopped 12 h prior to this examination.

Statistical analysis

Results are expressed as mean±SD. Serum IgE levels are given as the geometric mean and geometric SD of the mean. This is the antilog of the SD of the log₁₀-transformed serum IgE levels. Groups were compared by one-way analysis of

variance for multiple comparisons. The amounts of LTB₄ and LTC₄ released by leukocytes during escalation and clinically controlled periods were compared using a paired t-test. Relationships between LT generation and log₁₀ D_{min} were tested by linear regression analysis.

A p-value of <0.05 was regarded as significant.

Results

The baseline characteristics of the controls and asthmatic patients are presented in table 1. The type and dosage of medication did not differ among the asthmatic groups. Serum IgE levels did not differ significantly between patients with SDIA and non-SDIA in either the atopic or nonatopic group. The FEV₁ and FEV₁/forced vital capacity (FVC) did not differ significantly between atopic and nonatopic patients by leukocytes from asthmatics.

Table 1 shows the amount of LTB₄ and LTC₄ generated by the peripheral leukocytes of asthmatic patients and control individuals on stimulation with the calcium ionophore A23187. The leukocytes of atopic and nonatopic asthmatics who were not receiving glucocorticoid therapy generated significantly more LTB₄ ($p<0.05$) and LTC₄ ($p<0.01$) than those of controls. In contrast the production of either LTB₄ or LTC₄ by leukocytes of patients with atopic or nonatopic asthma treated with long-term systemic glucocorticoid therapy (SDIA) and those of controls did not differ significantly. Among asthmatics who were not receiving oral glucocorticoid therapy, significantly more LTC₄ was produced by the atopic than by the nonatopic asthmatic patients ($p<0.01$). The leukocytes of atopic asthmatics who were not on an oral glucocorticoid regimen generated significantly more LTC₄ than those of atopic patients with SDIA ($p<0.01$). The amounts of LTB₄ generated by patients with and without SDIA did not differ significantly.

The amount of LTB₄ generated by leukocytes of atopic asthmatic patients was 84.6 ± 51.6 ng·5 × 10⁶ cells⁻¹ during exacerbations, and 72.5 ± 30.0 ng·5 × 10⁶ cells⁻¹ when clinically controlled. Nonatopic patients generated 85.9 ± 34.5 ng·5 × 10⁶ cells⁻¹ during exacerbations, and 75.9 ± 31.6 ng·5 × 10⁶ cells⁻¹ when clinically controlled. The amount of LTB₄ generated during either period did not differ significantly between atopic and nonatopic asthmatics (fig. 1a).

Figure 1b shows the production of LTC₄ by the leukocytes of patients with atopic and nonatopic asthma in relation to asthma exacerbations. Significantly more LTC₄ was produced during the exacerbation period than when clinically controlled, by both the atopic and nonatopic asthmatics. The amount of LTC₄ generated by leukocytes from atopic asthmatics increased from 49.9 ng·5 × 10⁶ cells⁻¹ when clinically controlled, to 72.5 ng·5 × 10⁶ cells⁻¹ during the exacerbation period ($p<0.01$). The amount of LTC₄ produced by nonatopic asthmatics also increased significantly from 22.9 ng·5 × 10⁶ cells⁻¹ when clinically controlled, to 60.8 ng·5 × 10⁶ cells⁻¹ during the exacerbation period.

The relationship between the levels of LTB₄ and LTC₄ produced by leukocytes and bronchial hyperresponsiveness to methacholine in asthmatics who were not receiving oral glucocorticoid therapy when they were clinically controlled were examined. Figure 2a shows a highly sig-

Fig. 1. – Generation of: a) leukotriene (LT) B₄; and b) LTC₄ by leukocytes of patients with atopic and nonatopic asthma in relation to exacerbation. Results are from 11 atopic and eight nonatopic asthmatics. Leukocytes were obtained from atopic and nonatopic asthmatics during exacerbations (+) and clinically controlled periods (-). Horizontal bars represent means.

nificant correlation between the level of LTB₄ generation and log₁₀ D_{min} ($r=-0.792$, $p<0.0001$). When the level of LTC₄ production was plotted against log₁₀ D_{min}, the correlation was still significant but with a much larger dispersion of data around the regression line ($r=-0.489$, $p=0.0025$) (fig. 2b).

Discussion

LTB₄ and the cys-LTs (LTC₄, LTD₄ and LTE₄) play important roles in the pathophysiology of the airways of patients with bronchial asthma. LTB₄ (as well as interleukin 8) acts as a chemoattractant/activator of neutrophils, and causes bronchial hyperresponsiveness and neutrophil accumulation in the airway [15]. Eosinophils and mast cells may synthesize LTC₄, neutrophils synthesize LTB₄, and macrophages may synthesize both LTC₄ and LTB₄ [16]. Increased production of LTC₄ is often accompanied

Fig. 2. – Relationship between: a) leukotriene (LT) B₄ ($r=-0.792$, $p<0.0001$); and b) LTC₄ generation ($r=-0.489$, $p=0.0025$) by leukocytes and bronchial hyperresponsiveness to methacholine of patients with atopic (●) and nonatopic asthma (○) who were not on oral glucocorticosteroid therapy when they were clinically controlled. Regression lines are shown. D_{min} : minimum dose of methacholine, *i.e.* the cumulative dose reached at the inflection point at which the reciprocal of respiratory resistance decreased linearly.

by eosinophil accumulation in the airway [17]. The amount of LTC₄ produced by eosinophils depends not only on the number of eosinophils but also on their degree of activation [18].

In the present study, the generation of LTB₄ and LTC₄ by leukocytes stimulated with calcium ionophore A23187 was studied in patients with asthma, in relation to IgE-mediated all asthma exacerbations, and bronchial hyperresponsiveness. The results showed that the leukocytes of asthmatics who are not on oral glucocorticoid therapy but not those of asthmatics with so-called SDIA, produce significantly more LTB₄ and LTC₄ than those of controls. Furthermore, the leukocytes of asthmatics generated significantly more LTC₄ during exacerbations, than when clinically controlled. This suggests that enhanced LTC₄ production is closely related to the pathophysiology of asthma attacks. These results are consistent with a report that showed enhanced LTC₄ production by activated eosinophils during antigen challenge [19]. In addition, SCHAUER *et al.* [20] showed that, upon stimulation with the ionophore A23187, the granulocytes of asthmatic children generated significantly more LTC₄ than those of healthy controls. They also demonstrated that the granulocytes of

patients with a history of severe asthma formed more LTC₄ than those of patients with less severe disease.

The present results also revealed that the leukocytes of patients with atopic asthma who were not receiving oral glucocorticoid therapy generated significantly more LTC₄ than those of patients with nonatopic asthma. These results suggest that LTC₄ production is more closely related to IgE-mediated allergy than to other asthmatic reactions. However, the leukocytes of patients with atopic or nonatopic asthma generated similar levels of LTB₄. Levels of LTB₄ generation both during exacerbations and when clinically controlled were similar in both atopic and nonatopic asthmatics.

This study demonstrates that the leukocytes of patients with relatively more severe hyperresponsiveness to methacholine produced significantly more leukotriene B₄ than those of patients who are less hyperresponsive. FUJIMURA *et al.* [15] demonstrated that interleukin-8 induces bronchial hyperresponsiveness as well as airway neutrophil accumulation in guinea-pigs *in vivo*, and that this may be partly mediated by the release of leukotriene B₄. Although hyperresponsiveness to methacholine also significantly correlated with leukotriene C₄ production, this correlation was weaker than that between methacholine hyperresponsiveness and LTB₄ production. Taken together, the results of the present study suggest that immunoglobulin E-mediated allergy participates predominantly in the production of leukotriene C₄, which is enhanced during asthma exacerbations. In contrast, enhanced production of leukotriene B₄ might lead to an increase in bronchial hyperresponsiveness in patients with asthma.

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