

Leukotriene B₄ and C₄ generation by blood leukocytes after *ex vivo* stimulation by Ca-ionophore and opsonized zymosan in children with atopic dermatitis

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The *ex vivo* release of leukotrienes B₄ (LTB₄) and C₄ (LTC₄) from the leukocytes of children with atopic dermatitis (AD) was evaluated after stimulation with Ca-ionophore and opsonized zymosan and compared with that of control children of similar ages. The blood eosinophil counts and total serum IgE levels in AD children were significantly higher than those in control children. The production of LTC₄, but not LTB₄, was significantly higher in AD children than in control children. There was a significant correlation between the relative blood eosinophil count and LTC₄ generation after stimulation with both Ca-ionophore and opsonized zymosan in all subjects. Calculations of the amount of LTC₄ produced per eosinophilic cell showed that there was no significant difference between cells from AD children and control children in terms of their ability to produce LTC₄. These findings suggest that the enhanced LTC₄ generation is due to increased numbers of eosinophils rather than to enhanced releasability of these cells.

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Leukotrienes (LTs), which are 5-lipoxygenase metabolites of arachidonic acid, have potent allergic and inflammatory properties. Since increased levels of LTB₄, LTC₄ and LTD₄ have been reported in the skin-blister fluid of patients with atopic dermatitis (AD) and in late cutaneous reactions [1, 2], these LTs are considered to be important mediators in the pathophysiology of AD. On the other hand, the enhanced release of chemical mediators from bone marrow-derived cells, such as histamine from basophils [3] and eosinophil cationic protein (ECP) from eosinophils [4], is thought to play an important role in the pathogenesis of AD. Enhanced release of LTB₄ and LTC₄ by peripheral blood leukocytes has also been seen in adult patients with AD [5, 6]. However, little data is available on the releasability of LTC₄ from eosinophils, which are the main source of LTC₄ produced by leukocytes [7, 8] and are known to be involved in the development of atopic diseases including AD [9]. No previous studies on the releasability of LTB₄ and LTC₄ in leukocytes

from patients with AD have examined the relationship between LT generation and the severity of AD.

The present study was undertaken with the aim of evaluating the production of LTB₄ and LTC₄ from the leukocytes of AD children, and in particular, the release of LTC₄ from eosinophils. We measured the production of Ca-ionophore and opsonized zymosan-induced LTB₄ and LTC₄ by peripheral blood leukocytes in children with AD and investigated the relationship between the blood levels of eosinophils and the severity of AD and the generation of LTB₄ and LTC₄.

Material and methods

The experimental group consisted of 23 children from the Gothenburg area aged 4/12–15 years (median 8.8 years) with AD or a history of AD. Patients were diagnosed as having mild, moderate, or severe AD based on established criteria [10] and were divided into 3 groups as follows: Group A,

Table 1. Percentages of blood neutrophils, monocytes, eosinophils and basophils and total serum IgE values (mean and range) in control children and children with atopic dermatitis (AD) of differing severity (for explanation see text).

	Controls	AD		
		(Group A)	(Group B)	(Group C)
Number of subjects	11	8	7	8
Neutrophils (%)	44.2	52.3	52.8	42.5
	58.1–28.5	42.3–61.0	42.8–62.0	15.0–58.9
Monocytes (%)	4.7	4.1	4.0	4.9
	1.5–6.2	0.5–6.0	2.0–5.2	3.1–7.0
Eosinophils (%)	3.5	6.3**	6.0*	9.8*
	1.7–8.0	3.1–10.6	3.4–12.1	2.2–28.2
Basophils (%)	0.9	0.7	0.7	0.8
	0–2.5	0.5–2.8	0.5–3.1	0–1.8
Serum-IgE (IU/ml)	99.2	449.3**	940.8**	531.6*
	2.3–470	65–1223	75–1910	11–1820

* $p < 0.05$ compared with controls.

** $p < 0.01$ compared with controls.

patients without AD symptoms but with a history of AD within the last 6 months; Group B, patients with mild AD currently present; Group C, patients with moderate and severe AD symptoms. The control group consisted of 11 healthy children aged 7/12–13 years (median 10.2 years) with negative histories of atopy; most of them were the children of staff members. Neither group took non-steroidal antiinflammatory drugs or oral steroids for at least 7 days before sampling.

Informed consent was obtained from each subject and/or parent. All the studies were approved by the Ethics Committee of the University of Gothenburg.

Preparation of cells

Peripheral blood from AD patients and healthy children was collected into tubes containing heparin. After centrifugation at $200 \times g$ for 20 min, the blood cell layer was mixed with 2% Dextran T-500 solution (Pharmacia Biotechnology, Uppsala, Sweden) containing 0.9% NaCl to separate leukocytes from erythrocytes. Forty minutes later, the supernatant was gently sucked off and centrifuged at $2000 \times g$ for 15 min. The pellets were suspended in 0.15 M NH_4Cl solution (pH 7.4) for 30 min at 4°C to lyse the remaining erythrocytes. After centrifugation at $2000 \times g$ for 15 min, the pellets were resuspended in Dulbecco's phosphate-buffered saline (PBS) (Sigma, St. Louis, MO) and the concentration of leukocytes was adjusted to 15×10^6 cells/ml.

The viability of the leukocytes was checked occasionally by trypan blue exclusion and was found to exceed 90%.

Cell stimulation

After pre-incubation at 37°C for 5 min, the leukocytes were incubated with $5 \mu\text{M}$ Ca-ionophore A23187 (Calbiochem-Behring, La Jolla, CA) or with opsonized zymosan for 5 min at 37°C . The opsonized zymosan was prepared as follows. Fresh human serum was supplemented with zymosan A (Sigma, St. Louis, MO) (10 mg/ml) and incubated for 30 min at 37°C . After centrifugation at $100 \times g$ for 10 min, the pellets were washed twice in PBS and suspended in Dulbecco's PBS (final concentration 5 mg/ml). Four volumes of cold methanol was added to stop the incubation. After centrifugation at $800 \times g$ for 10 min at 4°C , the cell-free supernatant was removed and stored at -80°C until the assays were performed.

LTB₄ and LTC₄ assays

LTB₄ and LTC₄ were analyzed using a high-performance liquid chromatography (HPLC) technique, following the method developed by Odlander et al. [11]. Reverse-phase HPLC was performed using a column (4.6×150 mm) packed with $5 \mu\text{m}$ particles of Ultrasphere C-18 (Beckman, Palo Alto, CA). The elution (1 ml/min) was performed with acetonitrile: methanol: water: acetic acid (30: 19: 50: 1, v/v/v/v) and the pH was adjusted to 5.6 with 5N NaOH. The UV-absorption of the eluate was monitored at 280 nm using a Pharmacia-LKB 2141 variable UV-detector counted to a Hewlett-Packard 3396 integrator.

Blood analyses

For absolute leukocyte and differential counts, cytochemical analysis with Technicon H system was performed. Total IgE was measured by a radioimmunoassay (Kabi Pharmacia Diagnostics).

Statistical analysis

The results were expressed as mean $\pm$ SD. Differences were determined by Mann-Whitney U test. Correlations were determined using the Spearman rank correlation test. Statistical significance was defined as p value less than 0.05.

Results

No significant differences in the blood neutrophil, lymphocyte, monocyte and basophil counts could be observed between the three AD groups and the control group (Table 1). The blood eosinophil count and total serum IgE level in each AD group were significantly higher ($p < 0.05$ – 0.01) than those in the control group.

Table 2. LTB₄ and LTC₄ production (mean $\pm$ SD) by blood leukocytes and LTC₄ production in relation to blood eosinophil count with stimulation by Ca-ionophore (Ca-I) and opsonized zymosan (OZ) in a control group and A, B, and C groups of children with atopic dermatitis (AD). For explanation see text.

		Controls	AD		
			(Group A)	(Group B)	(Group C)
Number of subjects		11	8	7	8
LTB ₄ (pmol/ 15 $\times$ 10 ⁶ cells)	Ca-I	357.7 $\pm$ 76.1	409.2 $\pm$ 54.2	383.4 $\pm$ 132.8	348.6 $\pm$ 61.8
	OZ	94.1 $\pm$ 44.9	103.8 $\pm$ 36.2	117.1 $\pm$ 45.2	115.1 $\pm$ 28.3
LTC ₄ (pmol/ 15 $\times$ 10 ⁶ cells)	Ca-I	100.6 $\pm$ 38.5	163.0* $\pm$ 52.0	149.4* $\pm$ 38.2	188.4** $\pm$ 71.3
	OZ	35.4 $\pm$ 18.3	56.9 $\pm$ 22.5	61.4* $\pm$ 27.6	68.3* $\pm$ 35.7
LTC ₄ /Eos (pmol/ 15 $\times$ 10 ⁶ cells/%)	Ca-I	30.9 $\pm$ 12.3	24.8 $\pm$ 13.4	24.4 $\pm$ 7.0	23.2 $\pm$ 12.5
	OZ	12.2 $\pm$ 8.1	7.9 $\pm$ 2.5	11.0 $\pm$ 6.0	7.8 $\pm$ 4.5

* $p < 0.05$ compared with controls.

** $p < 0.01$ compared with controls.

The production of LTB₄ by blood leukocytes after stimulation with Ca-ionophore and opsonized zymosan did not differ between the groups (Table 2). In contrast, the generation of LTC₄ by the leukocytes after stimulation with Ca-ionophore in the three AD groups and after opsonized zymosan in the groups with active AD was significantly higher ($p < 0.05$ – 0.01) than that of the control group. There was a tendency towards higher LTC₄ levels in patients with severe disease, although the differences did not reach statistical significance. The LTC₄ generation did not, however, differ between each AD group and the control group when correlated to the relative blood eosinophil count. As a result, there was no indication that the production of LTC₄ per eosinophilic cell differed between the groups.

There was a significant correlation between the relative blood eosinophil count and LTC₄ production after stimulation with both Ca-ionophore ($r = 0.72$, $p < 0.01$) and opsonized zymosan ($r = 0.64$, $p < 0.01$) in all subjects (Fig. 1). As seen in the figure, a doubling of the eosinophil count resulted in an increase in the amount of LTC₄ produced of about 50%.

Discussion

This study showed that the production of LTC₄, but not LTB₄, by the peripheral blood leukocytes of AD children after stimulation with Ca-ionophore and opsonized zymosan was markedly increased compared with that of normal non-allergic children. The

blood levels of eosinophils, which are the major source of LTC₄ produced by blood leukocytes [7, 8], were also significantly higher in children with AD than in control children. In addition, there was a tendency towards higher LTC₄ and eosinophil levels in patients with severe AD. Several studies have demonstrated enhanced production of LTC₄ by isolated eosinophils in patients with asthma [7, 12, 13]. In the present study, it was not possible to evaluate the generation of LTC₄ by isolated eosinophils because of practical difficulties involved in obtaining sufficient volumes of blood from small children to isolate the required quantities of eosinophils. However, our results demonstrated a significant correlation between the relative blood eosinophil counts and the production of LTC₄ by peripheral blood leukocytes from AD and healthy children, whose eosinophil counts varied widely. This finding agrees with previous findings which indicate that eosinophils are the major source of LTC₄ [7, 8], and suggests that it would be possible to assess the LTC₄ production capacity of blood leukocytes simply by determining the blood eosinophil count. In our study, the generation of LTC₄ by peripheral blood leukocytes in relation to relative eosinophil count did not differ in AD children from that in control children. We therefore assume that there is no enhanced releasability of LTB₄ and LTC₄ from peripheral blood leukocytes in children with AD.

Previous studies which investigated the releasability of LTs from the peripheral blood leukocytes of patients with AD have shown that there is no significant difference in LTB₄ and LTC₄ release from leukocytes, monocytes and neutrophils between AD patients and controls after stimulation with Ca-ionophore [5, 6, 14]. This is in accordance with our results. Enhanced release of LTB₄ and LTC₄ from leukocytes and neutrophils has been observed following stimulation with anti-IgE, C5a, interleukin-3 and -8, and formyl-methionyl-leucyl-phenylalanine in patients with AD [5, 6]. This discrepancy in LT generation might be explained by the difference in stimuli. Non-physiological stimulation with Ca-ionophore is not thought to imitate *in vivo* stimulation. We did not, however, observe any enhanced release of LTB₄ and LTC₄ from the blood leukocytes of AD children after stimulation with opsonized zymosan, which is considered to be a physiological stimulus for LT generation. The reason for the contradictory results relating to LT releasability from the peripheral blood leukocytes of AD patients in our study in comparison with former reports is unclear, but we cannot dismiss the possibility that differences in the type of stimuli and in the age of patients may be the reason. Ruzicka et al. [5] did not evaluate LTC₄ production from leukocytes by determining blood eosinophil counts. Neuber et al. [6]

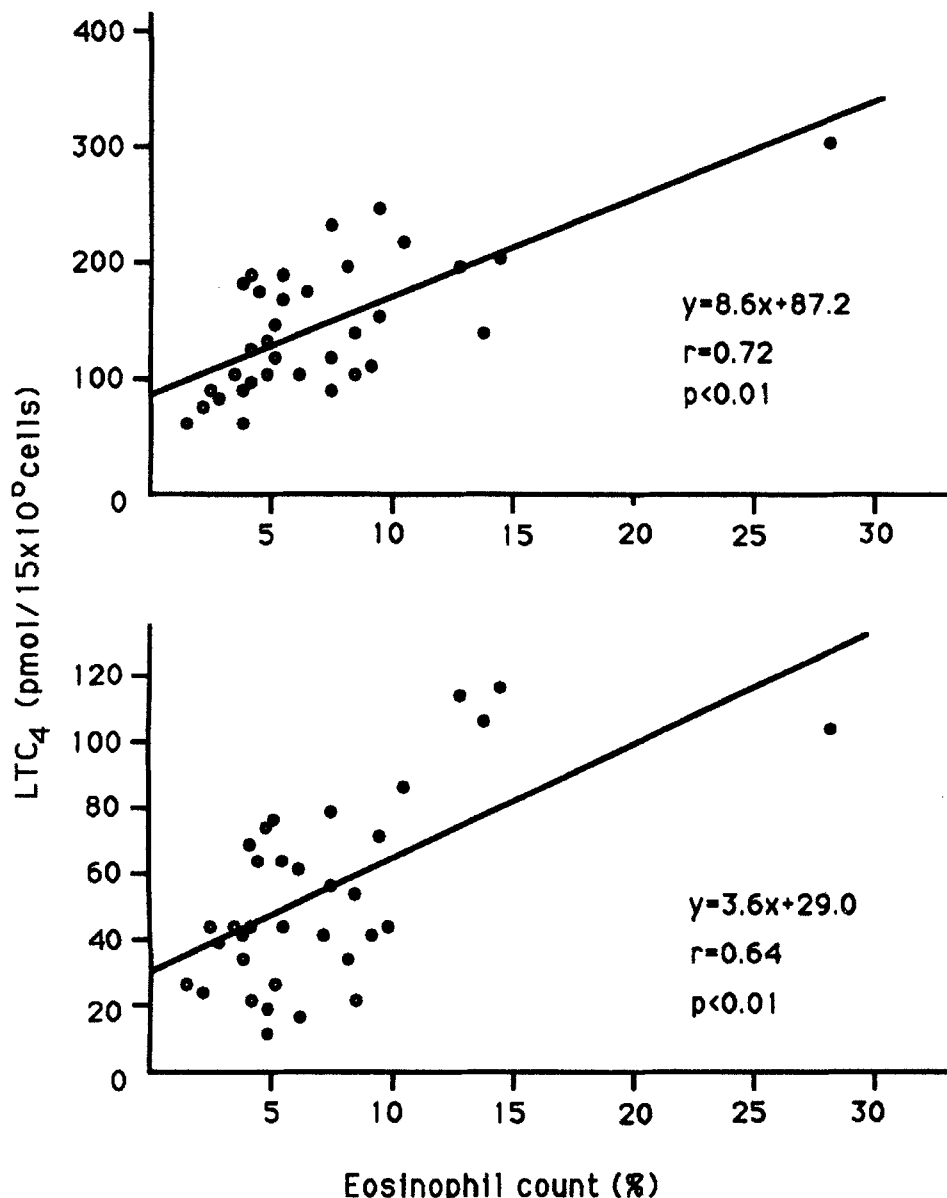


Fig. 1. Relationship between blood eosinophil count and LTC₄ generation by leukocytes from 11 control children (○) and 23 children with atopic dermatitis (●) after stimulation with Ca-ionophore (upper part) and opsonized zymosan (lower part).

measured LTC₄ generation by neutrophils, which are not an adequate source of LTC₄. Regarding the releasability of LTC₄, these factors may explain the contradictory results.

LTB₄ has potent chemokinetic and chemotactic effects on polymorphonuclear leukocytes [15] and leads to dermal infiltration and enhanced vascular permeability in the skin [16]. LTC₄ has potent vasoactive actions [15] and stimulates fibroblast proliferation [17]. Increased skin levels of these LTs have been reported in patients with AD [1] and late cutaneous reactions [2]. Thus, the pathophysiology of AD may partly be explained by the actions of increased LTB₄ and LTC₄ in the skin caused by both allergic and inflammatory responses, although the relationship between allergic reaction and leukotrienes in the skin is controversial [18, 19]. In the present study, we did not observe any enhanced

releasability of LTB₄ and LTC₄ from the peripheral blood leukocytes of AD children after stimulation with non-physiological and physiological stimuli. The increased generation of LTC₄ in AD children could be totally accounted for by the increased eosinophil counts in these patients. We, therefore, suggest that the high levels of LTB₄ and LTC₄ in the skin of AD patients are caused by an increased number of LT-producing cells and not by enhanced LT releasability of these cells. This has to be confirmed by studies of LT production by isolated cells from the skin of AD patients after stimulation with adequate immunological stimuli, such as opsonized zymosan, immunoglobulins, complements and cytokines.

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