

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

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The *ex vivo* release of leukotriene B₄ (LTB₄) and leukotriene C₄ (LTC₄) from leukocytes was evaluated after stimulation with both Ca-ionophore (Ca-I) and opsonized zymosan (OZ) in children with atopic asthma. Twenty-seven patients with asthma of varying severity were evaluated and divided into three groups: 1) moderate to severe asthma using inhaled steroids and symptom-free for the last 3 weeks (n = 8), 2) mild asthma with sporadic symptoms, only using inhaled β_2 -agonists < 3 times/week (n = 8), and 3) acute asthmatic attacks admitted to hospital (n = 11). A group of children without atopic disease or any other known disease served as controls (n = 15). Total serum IgE levels were significantly increased in the children with asthma compared with the control group. LTC₄ production was only significantly increased in the group of children with moderate to severe asthma after stimulation with Ca-I, when compared with controls. In the same group, a trend towards increased LTC₄ production after stimulation with OZ was found. LTB₄ was not significantly increased in any patient group compared with the control group. A significant correlation between LTC₄ production after stimulation with Ca-I, but not OZ, and the relative blood eosinophil count was found in all subjects. LTC₄ generation per eosinophilic cell after stimulation with Ca-I or OZ was not statistically different in any patient group compared with the controls. We conclude that the increased leukotriene (LT) levels found after the stimulation of peripheral white blood cells sampled from atopic children with asthma are mainly the result of increased numbers of LT-producing cells, rather than due to increased releasability from these cells.

**S. Kristjánsson, T. Shimizu,
G. Wennergren, I.-L. Strannegård,
B. Strandvik**

Department of Paediatrics, University of
Göteborg, Sweden

Sigurdur Kristjánsson, Department of Paediatrics,
University of Göteborg, Östra University Hospital,
S-41685 Göteborg, Sweden

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The cysteinyl leukotrienes (leukotriene C₄ (LTC₄), leukotriene D₄ (LTD₄), and leukotriene E₄ (LTE₄)), which are derived from arachidonic acid, were originally described in 1940 as the slow-reacting substance of anaphylaxis (1). The chemical structures of LTC₄ and LTD₄ were described four decades later (2, 3). Arachidonic acid is converted via leukotriene A₄ to leukotriene B₄ (LTB₄) or LTC₄ through the 5-lipoxygenase enzyme complex. LTC₄ is metabolized to its more stable end products LTD₄ and LTE₄.

Interest in LTs in relation to asthma is based on the known biochemical and pharmacological effects of these compounds (4–6) such as bronchoconstric-

tion, increased mucus secretion, changes in pulmonary vascular permeability and impaired mucociliary clearance. When it comes to bronchoconstriction, inhaled LTC₄ and D₄ have been shown to be more than 1000 times more potent than histamine in compromising airway lung function measured as the maximum expiratory airflow rate at 30% of vital capacity (7). For this reason, 5-lipoxygenase inhibitors or leukotriene receptor antagonists are regarded as promising for the treatment of asthma. LTB₄ is a very powerful chemotactic agent for human leukocytes and could therefore play an important role in the inflammatory reaction in the airways of patients

with asthma, although the role of LTs in asthma has not been established (9). In atopic patients with asthma symptoms, LTB_4 and LTC_4 have been found to be significantly increased in bronchoalveolar lavage (BAL) when compared with healthy controls (10). However, in another study, patients with bronchial hyperresponsiveness, atopy and mild asthma had levels of LTC_4 , D_4 , and E_4 in BAL fluid which were similar to those found in healthy non-asthmatic controls, while the LTB_4 levels were in fact lower (11). In children with atopy and exercise-induced asthma (EIA), an increase in urinary LTE_4 has been found after exercise challenge, when compared with healthy controls (12). Significantly increased urinary LTE_4 levels (exercise-induced) have been found in atopic children with severe EIA but not in children with moderate EIA (13).

No study has investigated children with asthma and atopic disease and their generation of LTB_4 and C_4 from peripheral leukocytes after stimulation with the potent stimulus Ca-ionophore (Ca-I) and the more "physiological" stimulus opsonized zymosan (OZ). The aim of this study was to investigate the generation of LTC_4 and LTB_4 , following stimulation with Ca-I or OZ, in peripheral blood leukocytes from children with atopic asthma of varying severity and treatment, in order to elucidate the possible influence of anti-asthma treatment on leukotriene generation.

Patients and methods

Forty-two children were included in the study. The study was conducted outside the pollen season between September 1991 and March 1992. Prior to inclusion, parental approval was given and a detailed medical history was obtained. The determination of asthma severity was based on the frequency of asthmatic symptoms and the need for treatment in order to remain symptom-free (14). All the subjects who were included were examined by the same doctor (S.K.) and a protocol was completed on the severity of asthma. Twenty-seven patients with asthma and atopy were divided into the following three groups according to the severity of asthma and the treatment: 1) Patients with moderate to severe asthma using inhaled steroids continuously (daily dose 400 μ g) for a period longer than 2 months and having been symptom-free for the last 3 weeks ($n = 8$), median age 11.2 (range 4.7–15.7) years; 2) patients with mild asthma with sporadic symptoms, using only inhaled β_2 -agonists < 3 times/week, but symptom-free when included ($n = 8$), median age 13.1 (7.3–16.0) years; 3) patients with acute asthmatic attacks admitted to hospital ($n = 11$), median age 6.8 (2.0–14.6) years. A control group was made up of children and schoolmates of children of staff work-

ing at the Department of Paediatrics. These children had no atopic diseases or any other known disease ($n = 15$) and had a median age of 10.4 (0.7–16) years.

Subject characteristics

There were no statistically significant differences in age between the groups. Atopy was defined by a positive skin prick test or serum RAST (Pharmacia Diagnostics) class 1 or higher, i.e., ≥ 0.35 kU/l, to one or more common allergens and increased levels of S-IgE. All the subjects, apart from the controls, fulfilled these criteria of atopy. Although some atopic patients had a history of atopic dermatitis, urticaria or allergic rhinoconjunctivitis, they were completely free from any signs of these complaints when they were examined.

All the patients in groups 1 and 2 (i.e., children with moderate to severe and mild asthma respectively) were subjectively healthy and, when questioned directly, stated that they had no current asthmatic symptoms. They had no signs of infection and physical examination revealed no rhonchi or other signs of asthma. Every patient on inhaled steroid treatment had a daily dosage of 400 μ g of either budesonide or beclomethasone. The patients with acute asthma were all suffering from asthmatic symptoms that had lasted for 1–20 days. All these patients were hospitalized because of asthma and treatment with bronchodilators and steroids, 2–300 mg hydrocortisone (Solucortef®, Upjohn) i.v. or 6 mg of betamethasone (Betapred®, Glaxo) p.o., had already been started 6–18 h prior to blood sampling. Most of the children with acute asthma had signs of respiratory tract infection of viral origin.

Blood analyses

Venous blood for the investigation of LTs was sampled after the application of local anaesthesia with lidocaine ointment (EMLA®, Astra) and collected in Vacutainer® SST tubes containing heparin. For absolute leukocyte and differential counts, a cytochemical analysis with Technicon H® system was performed. Total IgE was measured by Pharmacia CAP System, Ige RIA (Pharmacia Diagnostics, Uppsala, Sweden).

Cell preparation

After sampling, the blood was centrifuged at 200 g for 20 min. The blood cell layer was then mixed with 2% Dextran T-500 solution (Pharmacia Biotechnology, Uppsala, Sweden) in 0.9% NaCl to separate leukocytes from erythrocytes. Forty minutes later, the supernatant was gently sucked off and cen-

Table 1: Blood leukocytes, neutrophil and eosinophil counts and serum levels of IgE are presented as median values with ranges in parentheses. A statistically significant difference versus controls is indicated by asterisks ($p < 0.05 = *$; $p < 0.01 = **$; $p < 0.001 = ***$). A non-parametric Mann-Whitney U-test was used

	Controls		Inhaled steroids		β_2 -agonists		Acute asthma	
Number of subjects	15		8		8		11	
Leukocytes $\times 10^9/l$	5.5	(4.0–12.1)	6.2	(4.3–9.7)	7.4	(5.5–8.8)	9.8**	(7.0–15.1)
Neutrophils $\times 10^9/l$	2.6	(1.9–4.7)	2.6	(2.1–5.7)	4.0*	(0.1–5.1)	6.4***	(4.1–13.0)
Eosinophils $\times 10^9/l$	0.2	(0.1–0.5)	0.4*	(0.1–0.7)	0.3	(0.1–1.3)	0.3	(0.1–1.6)
IgE, kU/l	22	(2–470)	320**	(80–1700)	480***	(110–3300)	555**	(14–6500)

trifuged at 2000 g for 15 min. The pellets were suspended in 0.15 M NH_4Cl solution (pH 7.4) for 30 min at 4°C in order to disperse traces of erythrocytes by means of haemolysis. After centrifugation at 2000 g for 10 min, the pellets were resuspended in Dulbecco's phosphate-buffered saline solution (PBS) (Sigma, St Louis, MO, USA) and the concentration of leukocytes was adjusted to 15×10^6 cells/ml. The leukocyte viability was checked at regular intervals by trypan blue exclusion and was consistently found to exceed 90%.

Cell stimulation

After incubation at 37°C for 5 min, the leukocytes were incubated with 5 μM Ca-ionophore A 23187 (Calbiochem-Behring, La Jolla, CA, USA) or with OZ for 5 min at 37°C. Fresh human serum was supplemented with zymosan A (Sigma, St Louis, MO, USA) (10 mg/ml) and incubated for 30 min at 37°C. After centrifugation at 100 g for 10 min, the pellets were washed twice in PBS and suspended in the Dulbecco's PBS (final concentration 5 mg/ml). Four volumes of cold methanol were added to stop incubation. After centrifugation at 800 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 analysed using the high-performance liquid chromatography (HPLC) technique, following the rapid and sensitive method developed by Odlander et al. (15). Reverse-phase HPLC was performed using a column (4.6 $\times$ 150 mm) packed with Ultrasphere 5 μm C-18 particles (Beckman, Palo Alto, CA, USA). 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 5 M NaOH. The UV-absorption of the eluate was monitored at 280 nm using a Pharmacia-LKB 2141 variable UV-detector connected to a Hewlett-Packard 3396 integrator which determined the total content of LTB₄ and LTC₄. LTB₄ and LTC₄ were identified and correlated to the retention times of injected standards. Samples from different groups were analysed in parallel and the assays

were blinded. The data presented are the results of single determinations. The recovery of LTB₄ was 87.2% (SD $\pm$ 4.6, $n = 5$), while that of LTC₄ was 84.1% (SD $\pm$ 6.8%, $n = 5$). The detection limits for the LTB₄ and LTC₄ assays were 9.8 pmol and 6.2 pmol, respectively. The results of duplicated analysis revealed a deviation of less than 5% and analyses performed within month revealed a deviation of less than 8%.

Statistical analysis

The StatView 4.01™ software package was used for the statistical analysis. The results are given as median values and the ranges are shown in parentheses unless otherwise stated. Differences between groups were analysed using the Mann-Whitney U-test. Correlations were determined using Pearson's correlation coefficient. The study was approved by the Ethics Committee at the Medical Faculty of the University of Göteborg.

Results

White blood cells and total IgE

In the group of children with acute asthmatic attacks, the leukocyte and neutrophil counts were significantly increased compared with the control group ($p < 0.01$ and $p < 0.001$, respectively). Children with mild asthma and using β_2 -agonists < 3 times/week had significantly increased neutrophil levels compared with the controls ($p < 0.05$).

The blood eosinophil counts were significantly increased in the group treated with inhaled steroids when compared with controls ($p < 0.05$). The levels of total serum IgE were increased in all the patient groups (Table 1).

Generation of LTB₄ and LTC₄

The production of LTC₄ by peripheral white blood cells after stimulation with Ca-I and OZ is shown in Fig. 1 and Fig. 2, respectively. Following stimulation with Ca-I, LTC₄ production was only significantly increased in the group of children with moderate-severe asthma and using inhaled steroids as therapy when compared with controls (Fig. 1) (p

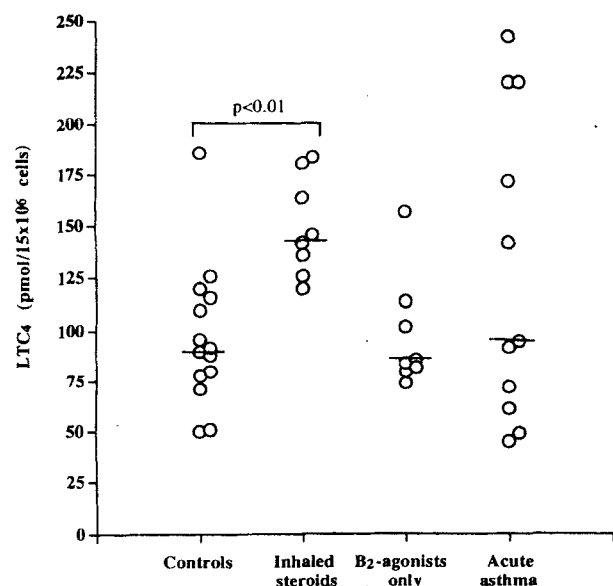


Fig. 1. LTC₄ values are shown for each group after stimulation with Ca-ionophore (○). Controls (n = 14), inhaled steroids (n = 8), β₂-agonists only (n = 8) and acute asthma (n = 11). Median values are indicated and significant differences compared with controls are shown. A non-parametric Mann-Whitney U-test was used for statistical comparison.

< 0.01). A trend towards increased LTC₄ production was also found in this group compared with the control group after stimulation with OZ (Fig. 2) (p = 0.06).

A significant correlation ($r = 0.5$, $p < 0.001$) between LTC₄ production after stimulation with Ca-I and the relative blood eosinophil count was found (Fig. 3). This was not seen following stimulation with OZ ($r = 0.1$, $p < 0.5$). A significant correlation

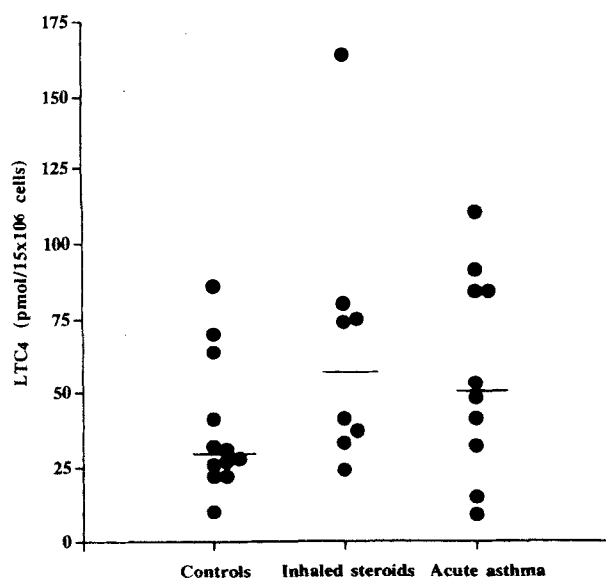


Fig. 2. LTC₄ values are shown for each group after stimulation with opsonized zymosan (●). Controls (n = 12), inhaled steroids (n = 8), acute asthma (n = 10). Median values are indicated.

between LTB₄ and the relative blood neutrophil count was found after stimulation with Ca-I ($r = 0.5$, $p < 0.001$, $n = 41$) and OZ ($r = 0.4$, $p < 0.01$, $n = 30$) LTB₄ generation and LTC₄ generation per eosinophil are shown in Table 2. Owing to technical errors, LTC₄ and LTC₄ was not measured after stimulation with OZ in the group of children receiving β₂-agonist therapy.

Discussion

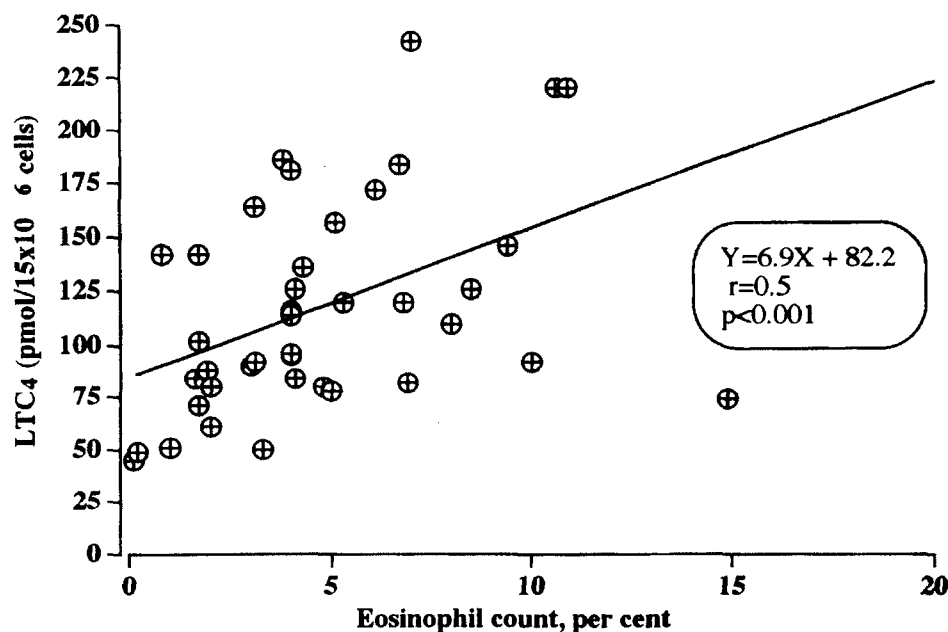
In this project, we used different stimuli to study the general LT production by the white blood cells of differently treated children with atopic asthma of varying severity. Ca-I was selected as a trigger because it is a potent stimulus of LTs at the concentration which we used and elicits a maximum metabolic response. The ionophore is thought to increase intracellular levels of calcium and directly to stimulate both release of arachidonic acid from phospholipid stores and to activate 5-lipoxygenase. OZ is thought to simulate a model for microbial invaders and to be a more physiological stimulus of leukotriene synthesis than Ca-I (16, 17). Zymosan itself activates the complement cascade, but on its own it is a weak stimulator for synthesis of LTB₄ and LTC₄. However, when zymosan is coated with serum it attaches to C3b and IgG and the induction of leukotriene synthesis is then thought to take place via the attachment to the C3b and Fc-receptors of human peripheral leukocytes (18). The levels of LTB₄ and LTC₄ after stimulation by OZ or IgG immune complexes have been shown to be 2–4 times lower than those found after stimulation with Ca-I (16).

No previous studies have compared LT generation in children after stimulation with Ca-I and OZ. The patient groups were carefully selected according to the severity of asthma and the treatment. In most previous studies, all drug treatment, especially the anti-inflammatory types, was discontinued 2–3 weeks before the blood was sampled. We decided not to do this, partly for ethical reasons, but also because we wanted to see whether ongoing treatment could possibly affect the release of LTs.

In our study, the children with moderate-severe asthma (having received inhaled steroids for 2 to 84 months) displayed a significant increase in the production of LTC₄ after stimulation with Ca-I. This could be explained by the elevated blood eosinophil counts, since there was a significant correlation between these two parameters.

This is in line with previous findings that eosinophils are the main source of LTC₄ (6, 19, 20). However, when the effect of OZ stimulation was evaluated, we did not find a similar overall linear correlation between an increase in eosinophil levels and

Fig. 3. The linear association between the percentage of blood eosinophils and LTC₄ production by white blood cells (after stimulation with Ca-I) from the whole group (n = 41) is shown. The percentage of eosinophils has been selected rather than the total number of eosinophils, because a fixed amount of leukocytes (15 × 10⁶ cells) was adjusted to a fixed volume (1 ml). The association between total eosinophil count and eosinophil percentage for the whole group was $r = 0.9$, $p < 0.001$.



LTC₄ generation. In another study of children with atopic dermatitis of varying severity who had no steroid treatment for at least 7 days before blood was sampled, however, we found a good linear correlation between LTC₄ generation and increased eosinophil counts after stimulation with both Ca-I and OZ (21). One possible explanation might be inhibitory effects on leukotriene synthesis by the steroids which were used in the group of children with acute asthma and inhaled steroids. However, this is not a convincing explanation because most previous studies have failed to demonstrate *in vivo* and *in vitro* effects by steroid treatment on leukotriene synthesis (22–23).

Although the levels of Ca-I-induced LTC₄ were significantly higher in incubations from steroid-treated atopic individuals, the amounts of LTC₄ generated by each eosinophilic cell were similar in patients and controls. This indicates that the increased LT levels that may be found in the body fluids of

atopic individuals are not a result of increased LT generation from individual cells but are instead due to increased numbers of LT-producing cells. Our results are in accordance with those of Balter et al. (24), who investigated cultured alveolar macrophages and their eicosanoid generation after Ca-I stimulation. These authors did not find any differences between the quantities or profiles of eicosanoid release by the cells of non-atopic controls and atopic, asthmatic subjects. LTB₄ is a powerful chemotactic factor for granulocytes (25) and is probably an important mediator at an early stage of the inflammatory reaction in asthmatic airways. The importance of LTB₄ in the pathogenesis of asthma is not clear. LTB₄ measured in arterial blood (26) and BAL fluid (10) has been shown to increase in symptomatic asthmatic patients, but Aizawa et al. (27) did not find any enhancement in the production of LTB₄ from neutrophils in asthmatic patients during symptom-free intervals. In our study the LTB₄ generation

Table 2: LTB₄ production by blood leukocytes after stimulation with a Ca-ionophore (Ca-I, upper part) and opsonized zymosan (OZ, lower part). LTC₄ production in relation to blood eosinophil count is shown. Results are given as median values with ranges within parentheses. A statistically significant difference compared with controls was not found. A non-parametric Mann-Whitney U-test was used

		Controls	Inhaled steroids	β ₂ -agonists	Acute asthma
Number of subjects		14	8	8	11
LTB ₄ pmol/15 × 10 ⁶ cells	Ca-I	322 (226–504)	386 (203–507)	302 (180–426)	370 (216–1129)
LTC ₄ pmol/15 × 10 ⁶ cells/eos %	Ca-I	30 (14–51)	24 (15–80)	24 (5–60)	29 (9–450)

		Controls	Inhaled steroids	Acute asthma
Number of subjects		12	8	10
LTB ₄ pmol/15 × 10 ⁶ cells	OZ	89 (48–196)	121 (40–184)	117 (22–248)
LTC ₄ pmol/15 × 10 ⁶ cells/eos %	OZ	13 (3–45)	8 (4–41)	8 (3–550)

was not significantly increased in any group, but we found a significant correlation between LTB₄ levels and the neutrophil counts after stimulation with both Ca-I and OZ. This indicates that the neutrophils are the main source of LTB₄ in blood (28).

We conclude that the increased LT levels found after the stimulation of peripheral white blood cells sampled from atopic children with asthma are mainly the result of increased numbers of LT-producing cells, rather than being due to increased releasability from these cells.

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