Exclusive leukotriene C_4 synthesis by purified human eosinophils induced by opsonized zymosan

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Received 19 July 1985

Purified human eosinophils were challenged with *N*-formyl-methionyl-leucyl-phenylalanine, leukotriene B_4, platelet-activating-factor, valyl-glycyl-seryl-glutamic acid, phorbol myristate acetate, zymosan, opsonized zymosan and the calcium ionophore A23187 to induce leukotriene synthesis. Reversed-phase high performance liquid chromatography analysis demonstrated the almost exclusive synthesis of leukotriene C_4 by eosinophils of 11 healthy donors after challenge with opsonized zymosan [$(22 \pm 4) \times 10^6$ molecules LTC4/cell, mean ± SE] or the calcium ionophore A23187 [$(54 \pm 7) \times 10^6$ molecules LTC4/cell, mean ± SE]. The other agents were not capable of inducing leukotriene formation. When in addition to opsonized zymosan *N*-formyl-methionyl-leucyl-phenylalanine or platelet-activating factor were added a significant increase of the leukotriene C_4 synthesis by eosinophils was observed. These results suggest that eosinophils might be triggered to produce considerable amounts of the spasmogenic leukotriene C_4 in vivo by C3b- and/or IgG-mediated mechanisms e.g. phagocytosis.

Leukotriene C_4 *Eosinophil* *Phagocytosis* *Opsonized zymosan* *Lipoxygenase* *Asthma*

1. INTRODUCTION

Recently, it has been shown that human eosinophils have the capacity to synthesize considerable amounts of the strongly bronchoconstrictive compound leukotriene C_4 (LTC4: 5(*S*)-hydroxy-6(*R*)-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) when stimulated in vitro with the calcium ionophore A23187 [1–3]. Since it has also been demonstrated recently that eosinophils infiltrate into the bronchioli at the beginning of the allergen-induced late-phase asthmatic reaction [4,5], both these findings throw new light on the role of eosinophils in the pathogenesis of asthma. As the late-phase asthmatic reaction is thought to be an inflammatory process, eosinophils might participate via C3b- and/or IgG-mediated mechanisms. In this study we present evidence that LTC4 formation by human eosinophils can be induced via C3b- and/or IgG-mediated mechanisms.

2. MATERIALS AND METHODS

2.1. *Materials*

Calcium ionophore A23187, reduced glutathione, phorbol-12-myristate-13-acetate (PMA), valyl-glycyl-seryl-glutamic acid (Val-Gly-Ser-Glu), *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), zymosan A, PGB2, 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy free radical were purchased from Sigma (St. Louis, MO). Ficoll-Paque (1.077 g/ml) and Percoll (1.129 g/ml) were obtained from Pharmacia (Uppsala, Sweden). Solvents, which were all of HPLC quality and octadecyl reversed-phase extraction columns (6 ml) were obtained from Baker (Phillipsburg, NJ). Synthetic LTB4, LTC4 and LTD4 were a kind gift of Dr J. Rokach (Merck-Frosst Laboratories, Pointe Claire/Dorval, Quebec, Canada). Human blood was obtained from healthy volunteers of the Red Cross Bloodbank Foundation (Utrecht).

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

2.2. *Preparation of opsonized zymosan (OZ) and zymosan-activated serum (ZAS)*

Freshly collected serum prepared of blood samples of 25 healthy volunteers was pooled and stored in fractions of 4 ml at −70°C until use. Zymosan was prepared as described by Robinson et al. [6]. Briefly: 500 mg of zymosan A is boiled in 50 ml phosphate-buffered saline (PBS) for 60 min. This suspension is washed twice with PBS. Batches of 75 mg boiled zymosan are suspended in 5 ml PBS and stored at −70°C until use. On the day of use, a batch of boiled zymosan is thawed and centrifuged (10 min, 1500 × g_{max}, room temperature). The pellet is resuspended in 3 ml of pooled normal serum and incubated for 30 min at 37°C. After centrifugation (10 min, 1500 × g_{max}, room temperature), the pellet (OZ) is resuspended in 5 ml Dulbecco's salt solution and kept cool (0°C) in the dark until use. The supernatant will be referred to as zymosan-activated serum (ZAS). OZ is used in a concentration of 5 mg/ml, unless otherwise stated. At this concentration no lactate dehydrogenase release could be observed.

2.3. *Purification of eosinophils*

Citrated blood was collected and platelet-rich plasma removed by centrifugation (15 min, 275 × g_{max}, room temperature). Granulocytes and mononuclear cells were separated by centrifugation of the buffy coat on Ficoll-Paque (20 min, 650 × g_{max}, room temperature). The mixed granulocytes were collected and the remaining erythrocytes removed by isotonic ammonium chloride lysis at 0°C and subsequent centrifugation. Thereafter the cells were regenerated at 37°C in minimum essential medium/10% fetal calf serum for 30 min (pH 7.4 at 37°C). After centrifugation the cells were resuspended in PBS (pH 7.4). The eosinophils were purified by subsequent centrifugation over isotonic Percoll layers with densities 1.082 g/ml and 1.085 g/ml as described [2,7]. Almost pure neutrophils appeared at the top of the Percoll solution with a density of 1.082 g/ml. The bottom layer consisted of an eosinophil enriched cell suspension. Further separation over a Percoll solution with a density of 1.085 g/ml resulted in an almost pure eosinophilic cell preparation on the bottom of the tube. Cell purities were generally over 85% and cell integrities over 95%.

2.4. *Incubation procedure and sample preparation*

Purified eosinophils were suspended in Dulbecco's salt solution (pH 7.4) at a concentration of 1×10^6 cells/ml, preincubated at 37°C for 5 min and then incubated for the indicated time period with a stimulant in the presence of 1 mM (extra) $CaCl_2$ and 5 mM reduced glutathione. Reactions were stopped by the addition of an equal volume of ice-cold water (for HPLC) or an equal volume of ice-cold LTC4-radioimmunoassay kit buffer (for RIA). Then the cells were spun down (20 min, 2000 × g_{max}, 4°C) and the supernatant analyzed. To samples for RP-HPLC analysis PGB2 was added as an internal standard, whereafter the samples were brought onto an octadecyl (C18) reversed phase extraction column. The adsorbed leukotrienes were eluted with 3 ml of methanol and stored under nitrogen at −70°C in the presence of a radical scavenger until analysis. Samples for RIA analysis were stored under nitrogen at −70°C until analysis.

2.5. *Analysis of leukotrienes*

2.5.1. By RIA

A commercially available LTC4-RIA (New England Nuclear, Boston, MA, USA) was used in accordance to the manufacturers instructions. LTC4 formation was routinely measured by RIA and additionally in some cases by RP-HPLC. LTC4 synthesis is expressed as the number of LTC4 molecules synthesized per cell (10^6 molecules LTC4/cell = 1.67 pmol LTC4/10^6 cells = 1.04 ng LTC4/10^6 cells).

2.5.2. By RP-HPLC

Leukotrienes were separated and quantified as described [8] using a CP Spher 10C18 column (250 × 4.6 mm, Chrompack, Middelburg, The Netherlands) attached to a Perkin-Elmer series 1 pump and a LC 85 detector. The solvent system was tetrahydrofuran-methanol-water-acetic acid (25:30:45:0.1, by vol.) which had been brought to pH 5.5 with ammonium hydroxide. The aqueous phase contained 0.1% EDTA to prevent binding of cations to the column. A flow rate of 0.9 ml/min was maintained and the effluent was monitored at 280 nm (leukotrienes, ϵ = 40000 $M^{-1} \cdot cm^{-1}$, PGB2, ϵ = 28650 $M^{-1} \cdot cm^{-1}$).

3. RESULTS

3.1. *LTC4 formation by eosinophils induced by OZ*

Isolated eosinophils were challenged for 30 or 60 min with the following agents to induce LTC4 synthesis: fMLP (1 nM–1 μM), LTB4 (0.1 nM–1 μM), PAF (10 nM–1 μM), Val-Gly-Ser-Glu (0.1 nM–1 μM), PMA (10 nM), ZAS (undiluted and dilutions 1:3 and 1:9), zymosan (0.1–5 mg/ml) and OZ (0.1–10 mg/ml). LTC4 synthesis could be observed only after challenge with OZ. When eosinophils were challenged for 30 min with OZ (5 mg/ml), (22 ± 4) × 10^6 molecules LTC4/cell are formed, whereas after optimal stimulation of the same cells (20 min) with A23187 (10 μM), (54 ± 7) × 10^6 molecules LTC4/cell were formed (n = 11, mean ± SE, cell purity, 86 ± 4%). The time course of LTC4 formation by isolated eosinophils when stimulated with OZ (5 mg/ml) is shown in fig.1. Based on the time course experiments an incubation time of 60 min was chosen to study the stimulant concentration dependence of the OZ-induced LTC4 formation. In fig.2 it is shown that zymosan particles as such are not capable of inducing LTC4 synthesis. OZ-induced LTC4 synthesis reaches a plateau after an OZ concentration of 2.5 mg/ml. When zymosan particles were opsonized with heat-inactivated serum (30 min at 56°C) a 90% decrease of the LTC4 formation was observed (n = 5).

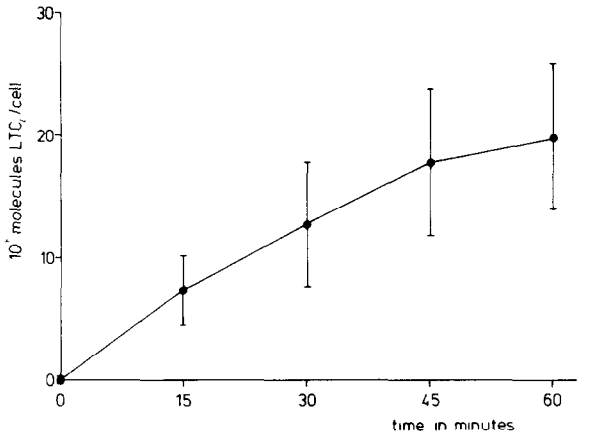

Fig.1. Time course of LTC4 formation by purified human eosinophils (purity, 84 ± 5%) upon stimulation with opsonized zymosan (5 mg/ml) (mean ± SE, n = 5).

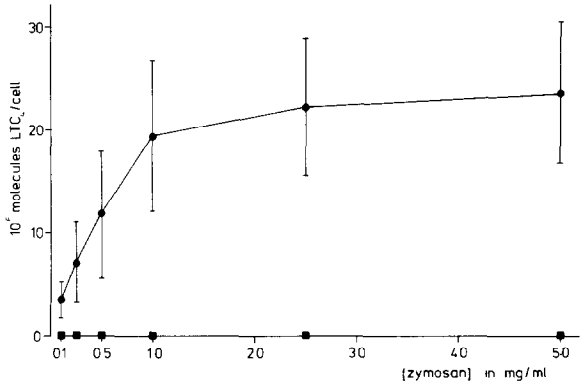

Fig.2. LTC4 formation by purified human eosinophils when stimulated for 60 min at 37°C with increasing amounts of zymosan particles ((■), mean ± SE, n = 3, purity of the eosinophils, 83 ± 3%) or opsonized zymosan particles ((●), mean ± SE, n = 7, purity of the eosinophils, 86 ± 3%).

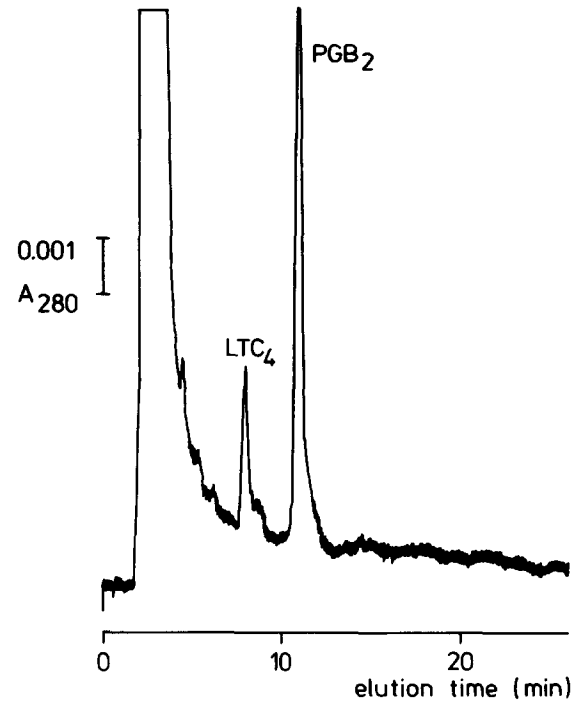

Fig.3. Illustrative example of a RP-HPLC chromatogram showing the exclusive formation of LTC4 by purified human eosinophils (purity, 87%) when stimulated with OZ + PAF for 30 min. LTC4 was identified by (1) RP-HPLC retention time, (2) coelution with synthetic LTC4 and (3) UV spectrum.

Table 1

LTC4 formation (in 10^6 molecules/cell) by human eosinophils after stimulation for 30 min with OZ (5 mg/ml), fMLP (100 nM), PAF (1 μM), LTB4 (50 nM), PMA (10 nM) or a combination of OZ with each of the other compounds

Stimulant	n	Cell purity (mean ± SE)	LTC4 formation (mean ± SE)	Paired t-test
OZ	14	84 ± 3	16 ± 3	$p < 0.001$
OZ + fMLP	14		24 ± 4	
fMLP	14		0.0 ± 0.1	
OZ	11	88 ± 2	18 ± 3	$p < 0.05$
OZ + PAF	11		24 ± 5	
PAF	11		0.7 ± 0.1	
OZ	6	90 ± 2	21 ± 4	n.s.
OZ + LTB4	6		24 ± 6[a]	
LTB4	6		0.3 ± 0.1	
OZ	4	90 ± 1	26 ± 4	n.s.
OZ + PMA	4		25 ± 3	
PMA	4		0.4 ± 0.2	

[a] Extension of the incubation time to 60 min resulted in a more pronounced stimulation

The OZ-induced LTC4 formation by eosinophils proved to be completely dependent on the presence of both reduced glutathione (5 mM) and $CaCl_2$ (2 mM) in the incubation medium (n = 3). Therefore stimulations were always performed in the presence of reduced glutathione (5 mM) and $CaCl_2$ (2 mM).

3.2. *Effect of various agents on the LTC4 formation by eosinophils induced by OZ*

LTC4 formation by eosinophils was measured after challenge for 30 min with fMLP (100 nM), PAF (1 μM), LTB4 (50 nM) and PMA (10 nM). Although these compounds as such did not induce LTC4 synthesis by eosinophils, the first two compounds were found to stimulate the OZ-induced LTC4 synthesis significantly as is shown in table 1.

RP-HPLC analysis showed that besides LTC4 no other leukotrienes are formed by eosinophils when stimulated with OZ alone or in combination with fMLP, LTB4, PMA or PAF (fig.3).

4. DISCUSSION

These results show that OZ is capable of inducing LTC4 synthesis by human eosinophils and illustrate that considerable amounts of LTC4 can be formed via C3b- and/or IgG-mediated mechanisms [9]. Since stimulation with zymosan, treated with heat-inactivated serum, was found much less effective than stimulation with OZ, it might be concluded that the C3b-receptor is more important in this process than the IgG-receptor. This is in agreement with the small stimulatory effect which has been reported for IgG-coated Sepharose-particles [10]. It has been shown that chemotactic factors like fMLP and PAF may increase the expression of IgG- and C3b-receptors on eosinophils [11–14]. This finding might explain the stimulatory effect of these compounds on the OZ-induced LTC4 formation by eosinophils. Consequently, PMA, which has no effect on the expression of C3b- and IgG-receptors, was found inactive in this respect.

In conclusion, this study shows that human eosinophils might be triggered to produce LTC4 by C3b- and/or IgG-mediated mechanisms (e.g. phagocytosis) and that certain chemotactic agents can amplify this effect.

ACKNOWLEDGEMENTS

L. Koenderman is thanked for typing the manuscript. This work was supported in part by grant 82.18 from the Netherlands Asthma Foundation.

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