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Effect of cations on leukotriene release: requirements for the metabolism of peptido-leukotrienes (leukotrienes C_4, D_4) by human polymorphonuclear granulocytes

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SUMMARY

Stimulation of human polymorphonuclear granulocytes with opsonized zymosan leads to a time-dependent release of the leukotrienes LTB_4, 6-trans-LTB_4, 12-epi-6-trans-LTB_4 and LTC_4 measured by HPLC analysis and LTC_4 radioimmunoassay. The amounts of leukotrienes released on stimulation with opsonized zymosan are lower than those obtained with the calcium ionophore A23187. Opsonized zymosan as stimulus required higher calcium concentrations to obtain optimal leukotriene release as compared with the calcium ionophore. Magnesium in the presence of calcium increased the leukotriene formation with opsonized zymosan. Addition of the peptido-leukotrienes LTC_4, LTD_4 to the unseparated, opsonized zymosan-prestimulated cell suspension leads to the generation of 6-trans-LTB_4, 12-epi-6-trans-LTB_4 and a metabolite which is more polar than LTC_4. The rate of LTC_4 metabolism is strongly dependent on the time of prestimulation as well as on the stimuli used for cell triggering, e.g. opsonized zymosan, phorbol-12-myristate-13-acetate, calcium ionophore A23187 or formyl-methionyl-leucyl-phenylalanine. Inhibitors of the oxidative burst decreased LTC_4 metabolism. Thus, the peptido-leukotriene LTC_4 is metabolized by two pathways: (i) the enzymes γ-glutamyl-transpeptidase and dipeptidase transform LTC_4 into LTD_4 and LTE_4; these enzymes are present within the supernatants of stimulated cells; (ii) transformation of LTC_4 into LTB_4-isomers occurs in the presence of stimulated cells.

INTRODUCTION

Leukotrienes are significantly involved in a variety of diseases, including asthma, inflammation and various allergic conditions (Bach, 1982; Borgeat & Sirois, 1981; Lewis & Austen, 1981). They are produced from arachidonic acid via a 5-lipoxygenase (Borgeat, Hamberg & Samuelsson, 1979; Borgeat & Samuelsson, 1979). The first step in their metabolism is the oxygenation of arachidonic acid at the C-5 position, leading to 5-hydroperoxyeicosatetraenoic acid (5-HPETE). This hydroperoxy compound is relatively unstable in aqueous solution; it is either reduced to 5-HETE or converted to the unstable allylic epoxide leukotriene A_4 (LTA_4). Neutrophils with the enzyme leukotriene A_4 hydrolase catalyse the hydrolysis of leukotriene A_4 to the chemotactic and chemokinetically active dihydroxy acid leukotriene B_4 (Rådmark *et al.*, 1980, 1984). The hydrolysis can also occur non-enzymatically and leads then to the formation of two LTB_4 isomers 5(S)12(R)-6-trans LTB_4 and 5(S)12(S)-6-trans-LTB_4 (Borgeat & Samuelsson, 1979). In human polymorphonuclear leucocytes, LTB_4 and its isomers are metabolized by omega oxidation (Powell, 1984; Shak & Goldstein, 1984). Alternatively, the enzyme glutathione-S-transferase can conjugate LTA_4 with reduced glutathione to produce leukotriene C_4. LTC_4 is then transformed by γ-glutamyl-transpeptidase into leukotriene D_4, which is further metabolized by dipeptidase activity into leukotriene E_4. The enzymatic conversion of LTC_4 into LTD_4 and LTE_4 has been shown in human plasma (Köller *et al.*, 1985), with RBL cell homogenate (Jakschik, Harper & Murphy, 1982), PMN homogenate fractions (Brom *et al.*, 1984) and in the supernatants of stimulated neutrophils (Raulf, Stüning & König, 1985). In addition, the inactivation of leukotriene B_4, C_4 and D_4 by eosinophil peroxidase (Henderson, Jörg & Klebanoff, 1982) and the conversion of leukotriene C_4 to isomers of leukotriene B_4 by this enzyme (Goetzl, 1982) have been described. A similar mechanism for the inactivation of

Abbreviations: 12-epi-6-trans-LTB_4, 5(S), 12(S)-dihydroxy-6,8,10,14-(EEEZ)-eicosatetraenoic acid; fMLP, formyl-methionyl-leucyl-phenylalanine; HOCl, hypochlorous acid; PBS, phosphate-buffered saline; PMA, phorbol-12-myristate-13-acetate; PMN, polymorphonuclear neutrophil leucocyte; RIA, radioimmunoassay; RP-HPLC, reverse-phase high performance liquid chromatography; SOD, superoxide dismutase; TLC, thin-layer chromatography; 6-trans-LTB_4, 5(S), 12(R)-dihydroxy-6,8,10,14-(EEEZ)-eicosatetraenoic acid.

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peptido-leukotrienes LTC_4 and LTD_4 to LTB_4 isomers and sulphoxides by the myeloperoxidase system and hydroxyl radicals was observed in neutrophils (Lee *et al.*, 1982; Henderson & Klebanoff, 1983a).

It is obvious that the inflammatory potential of granulocytes reflects the actual concentration of mediators resulting from both synthesis and deactivation. In the past, we have demonstrated that an eosinophil chemotactic factor (ECF) of low molecular weight, which was identified as LTB_4, was generated and released from human polymorphonuclear neutrophils (Czarnetzki, König & Lichtenstein, 1976; König, Frickhofen & Tesch, 1978) and rat mast cells (Kroegel, Kunau & König, 1981) by the calcium ionophore A23187 during phagocytosis of opsonized zymosan (König, Czarnetzki & Lichtenstein, 1976), by the bee venom peptide melittin (Kroegel *et al.*, 1981) and by bacterial exo- and endotoxins (Bremm *et al.*, 1984a, b).

Since it has been shown that granulocytes are capable of synthesizing as well as inactivating leukotrienes, interest has been focused on the biochemical mechanism responsible for the induction and metabolism of leukotrienes.

It was the purpose of the present investigation to analyse the requirements for leukotriene release induced by various stimuli. Evidence will be presented to suggest that various stimuli differ in their potency to induce leukotriene release and initiate their metabolism.

MATERIALS AND METHODS

Reagents

Reagents used were from the following sources: Zymosan A, Caionophore A23187, heparin, fMLP, PMA, catalase (from bovine liver 2000–5000 U/mg protein), SOD (3000 U/mg protein) and L-serine were obtained from Sigma Chemical Co., Taufkirchen, FRG; Ficoll 400 was from Pharmacia, Uppsala, Sweden; Macrodex 6% (w/v) was from Knoll, Ludwigshafen, FRG; sodium metrizoate solution 75% (w/v) was from Nyegaard, Oslo, Norway. [^{3}H]LTC_4 (specific activity 35·7 Ci/mol), [^{3}H]LTD_4 (specific activity 39·0 Ci/mol), [^{3}H]LTE_4 (specific activity 39·0 Ci/mol) and [^{3}H]LTB_4 (specific activity 32·0 Ci/mol) were from New England Nuclear, Dreieich, FRG. Synthetic leukotrienes C_4, D_4, E_4 and B_4 were kindly provided by Dr J. Rokach (Merck Frosst, Pointe Claire, Québec, Canada), and 6-trans-LTB_4 and 12-epi-6-trans-LTB_4 were from Dr P. Borgeat (L'Université Laval, Québec, Canada).

Buffer

The buffer used throughout all experiments consisted of 0·137 M NaCl/0·008 M Na_2HPO_4/0·0027 M KH_2PO_4 and 0·0027 M KCl, pH 7·4 (modified Dulbecco PBS; referred to as PBS).

Preparation of cells

Human PMNs were obtained from 200 ml of heparainized blood (15 U/ml) of healthy donors separated on a Ficoll–metrizoate gradient followed by dextran sedimentation (Böyum, 1968). In order to remove the platelets, the cells were washed twice at low speed. This method led to more than 95% pure and intact PMNs. The neutrophils were resuspended to a final concentration of 2×10^7 cells/ml in PBS.

Preparation of opsonized zymosan

Zymosan A (2 mg/experimental tube) was suspended in PBS (2 ml) and boiled for 5 min. PBS (15 ml) was added and the suspension was centrifuged for 10 min at 4° and at 1900 *g* (Heraeus, Christ, Cryofuge 6). The pellet was washed twice with PBS, resuspended in human serum (10 mg/ml human serum) and incubated for further 30 min at 37°. After centrifugation, the pellet was washed twice with PBS.

Stimulation of PMN

PMNs (1×10^7/0·5 ml) were incubated with freshly prepared opsonized zymosan (2 mg/0·2 ml PBS) in the presence of 1·25 mM calcium and 3·125 mM magnesium for 30 min or for various times as indicated. In several experiments, the cells were stimulated with opsonized zymosan at different concentrations of calcium (0–12·5 mM) or magnesium (0–12·5 mM), or with a mixture of 1·25 mM calcium and different magnesium concentrations (0–12·5 mM). Cells (1×10^7) stimulated with Ca ionophore A23187 were incubated for 15 min at 37° with the Ca ionophore ($7{\cdot}3 \times 10^{-6}$ M) in the presence of different calcium concentrations (0–8·2 mM).

Metabolism of exogenously added LTC_4 and LTD_4 by prestimulated cells

PMNs (1×10^7, 500 µl) were preincubated with opsonized zymosan (2 mg), Ca ionophore ($1{\cdot}6–16 \times 10^{-6}$ M), fMLP ($0{\cdot}2–2 \times 10^{-4}$ M) or PMA ($2{\cdot}5 \times 10^{-6}$ M) in the presence of 1·25 mM calcium and 3·125 mM magnesium for different times at 37°. After preincubation with the stimulus, LTC_4 or LTD_4 (150 ng in 50 µl buffer) or the same volume of PBS without leukotrienes was added to the cell suspension, and incubation proceeded for an additional 15 min (unless stated otherwise). Samples receiving PBS instead of synthetic leukotrienes served as controls in order to determine the active release of leukotrienes. The reaction was stopped by centrifugation in the cold at 400 *g*. The cell-free supernatants were deproteinized and extracted for HPLC (method described above). The reaction mixtures were also analysed by the addition of labelled leukotrienes (0·1 µCi [^{3}H]LTC_4 or [^{3}H]LTD_4 per experimental tube; specific activity was diluted to 1·2 Ci/mol with synthetic unlabelled leukotrienes); the analysis was then carried out by thin-layer chromatography.

Analysis of leukotrienes

RP-HPLC. For analysis of leukotriene release and their metabolism, the cell-free supernatants were deproteinized by the addition of 2 ml of methanol/acetonitrile (50:50, v/v) overlayered with argon and frozen at −70° for 12 hr. After centrifugation at 1900 *g* for 10 min at 4°, the supernatants were evaporated to dryness by lyophilization (EF4 Modylo, Edwards-Kniese). The residues were dissolved in 600 µl of methanol/water (30:70, v/v), covered with argon and left at −20° for 2 hr. Centrifugation was performed at 9700*g* for 2 min at room temperature (Eppendorf centrifuge 3200); the supernatants were then applied to HPLC. HPLC analysis was performed on reverse-phase columns (4 × 200 mm, packed with Nucleosil, 5-µm particles, Machery Nagel, Düren, FRG) with methanol:water:acetic acid (64:36:0·08, v/v adjusted to pH 5·7 with ammonia; the aqueous phase contained 0·05% EDTA) as eluent. For identification of recorded HPLC peaks, the α-factors, which were defined as the ratio of k' values (capacity ratios) of two peaks, were calculated. The α-factor (relative retention) represented the ratio of the k' value of an unknown substance to a reference substance. For the analysis of leuko-

triene release, LTB_4 served as reference substance to calculate the α-factors of the other substances. The calculated α-factors were: 0·53–0·56 for LTC_4, 0·74–0·77 for 6-trans-LTB_4 and 0·85–0·88 for 12-epi-6-trans-LTB_4 and LTD_4. When the metabolism of exogenously added leukotrienes was studied, the corresponding leukotriene that was added to the cell suspension was used as a reference substance. The quantification of identified leukotrienes was performed by area integration of the absorption peaks. With the described extraction procedure, the recovery rates of leukotrienes from 800 μl cell suspension were 75–85% for the peptido-leukotrienes and 90–95% for LTB_4.

Thin-layer chromatography. The cell suspensions were incubated with [^{3}H]LTC_4 or [^{3}H]LTD_4; subsequently, the reaction was stopped by adding 2 ml of methanol/acetonitrile (50:50, v/v); the samples were frozen at −70° for 12 hr. The denatured protein and cell debris were removed by centrifugation (1900 ***g***, 10 min, 4°), and the clear supernatants were evaporated to dryness under nitrogen. The residues were dissolved in 400 μl of methanol/water (30:70, v/v) and left at −20° for 1–2 hr. After centrifugation for 2 min at 9700 ***g***, the supernatants were evaporated to dryness under a gentle stream of nitrogen. The residues were dissolved in 40 μl methanol/water (30:70, v/v) and spotted on a silica gel thin-layer plate (250 μm, Kieselgel 60, Merck, Darmstadt, FRG). The plates were developed once with a solvent system: butanol/water/pyridine/acetic acid (30:24:20:6, v/v) for 4 hr at room temperature. [^{3}H]LTC_4, [^{3}H]LTB_4, [^{3}H]LTD_4 and [^{3}H]LTE_4 served as references. Their R_f values (mean ± SEM from $n=8$ experiments) were: [^{3}H]LTC_4: $R_f=0{\cdot}51\pm0{\cdot}01$, [^{3}H]$LTD_4$: $R_f=0{\cdot}65\pm0{\cdot}01$, [^{3}H]$LTE_4$: $R_f=0{\cdot}73\pm0{\cdot}02$ and [^{3}H]LTB_4: $R_f=0{\cdot}90\pm0{\cdot}01$. The applied conditions were not suitable for the separation of LTB_4 from its isomers. Radioactivity was detected with an Isomess Radio-Dünnschicht-Analysator IM 3000 (Isotopenmeßgeräte GmbH, Straubenhardt, FRG). The losses of radioactivity present within the two pellets were determined by liquid scintillation counting (Packard Tri-Carb Liquid Scintillation Spetrometer 3255, external standardization); combined, they accounted for 10–15%.

Radioimmunoassay. Aliquots (200 μl) of the deproteinized cell supernatants were evaporated to dryness under a stream of nitrogen and resuspended in 400 μl Tris-HCl buffer (pH 7·4, 0·01 M with 0·14 M NaCl and 0·1% gelatine). Three different dilutions were then applied to the LTC_4 radioimmunoassay. For the RIA, an appropriate anti-plasma dilution, as well as either standard LTC_4 (10 ng–25 pg) or the unknown diluted samples, were added to tubes containing [^{3}H]LTC_4 (15, 000 d.p.m.) in a total volume of 0·6 ml; incubation proceeded for 2 hr at 37°. Antibody-bound and free ligands were separated using 0·5 ml charcoal suspension (20 mg/ml) and subsequent precipitation by centrifugation. The supernatants (0·9 ml) were added to 9 ml Rotiszint 2200 (Roth, Karlsruhe, FRG) and the radioactivity was determined in a liquid scintillation counter. All quantifications by RIA were carried out as triplicates.

Determination of marker enzymes

Lysozyme (EC 3.2.1.17), β-glucoronidase (EC 3.2.1.31) and lactate dehydrogenase (LDH) (EC 1.1.1.27) were determined as previously described (König *et al.*, 1978).

RESULTS

Human polymorphonuclear leucocytes (1×10^7) stimulated with opsonized zymosan (2 mg/1×10^7 PMN) released maximal levels of leukotrienes into the supernatants after 20 min at 37° as was detected by RP-HPLC. The metabolites generated were identified as LTB_4, 6-trans-LTB_4 and 12-epi-6-trans LTB_4. LTB_4 proved to be the main product. Under identical incubation conditions, opsonized zymosan as stimulus released about 15% ($22{\cdot}1\pm0{\cdot}6$ ng LTB_4/1×10^7 PMN, $n=3$) of the amount of LTB_4 as compared to ionophore ($7{\cdot}3\times10^{-6}$ M)-stimulated cells (190 ± 26 ng LTB_4/1×10^7 cells, $n=17$). When supernatants of cells stimulated with opsonized zymosan were analysed with a radioimmunoassay for LTC_4, LTC_4 reached an average value of 1·5 ng/1×10^7 PMN (mean of three experiments). In contrast, stimulation of PMNs with the Ca ionophore ($7{\cdot}3\times10^{-6}$ M) led to the release of 53 ± 11 ng LTC_4/1×10^7 cells ($n=17$) as was analysed by HPLC and RIA for LTC_4. Stimulation of PMNs (1×10^7) with fMLP (2×10^{-5} M) in the presence of calcium (0·83 mM) induced a maximal release of $10{\cdot}2\pm5{\cdot}2$ ng LTB_4 ($n=3$) already after 5 min of incubation, as was quantified by HPLC.

Figure 1 shows the results of three separate time-course experiments with opsonized zymosan (2 mg/1×10^7 PMN) in the presence of 1·25 mM calcium and 3·125 mM magnesium as stimulus; LTB_4 was released after 3 min of incubation and reached a maximum of $22{\cdot}1\pm0{\cdot}6$ ng LTB_4/1×10^7 PMN after 20 min of incubation. With longer times of incubation a decrease in LTB_4 release is observed. LTC_4 reached a maximum of $2{\cdot}3\pm0{\cdot}99$ ng/1×10^7 PMN by 60 min of incubation.

We then studied the dependence of LTB_4-release on the presence and concentration of divalent cations such as calcium and magnesium. In preliminary experiments, human granulocytes were stimulated in the absence of calcium but in the presence of various magnesium concentrations (0·125–12·5 mM) with opsonized zymosan for 30 min at 37°. Neither LTB_4 nor LTC_4 release was detected by HPLC and LTC_4-RIA. Similar results were obtained at low calcium concentrations (0·125–1·0 mM) (Fig. 2). With calcium concentrations above 1·0 mM LTB_4 release was detected by HPLC. Four separate dose experiments (Fig. 2) showed a maximum of $64\pm13{\cdot}4$ ng/1×10^7 PMNs ($n=4$) for LTB_4 at a calcium concentration of 6·25 mM. A similar release pattern as for LTB_4 was observed for 6-trans-LTB_4 and 12-epi-6-trans-LTB_4. A decrease in LTB_4 release was obtained with calcium concentrations higher than 6·25 mM [at a

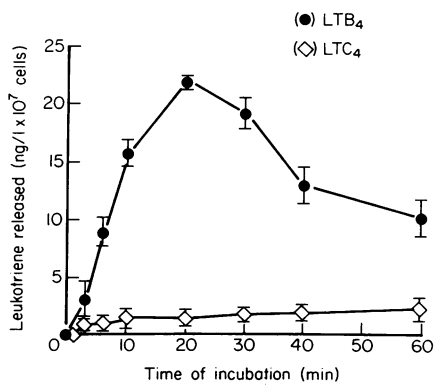

Figure 1. Time-dependent release of LTB_4 and LTC_4 from human PMNs on stimulation with opsonized zymosan. LTB_4 release was analysed by HPLC, and LTC_4 release was measured by RIA. Each value represents the mean ± SEM of three independent experiments performed with different donor cells.

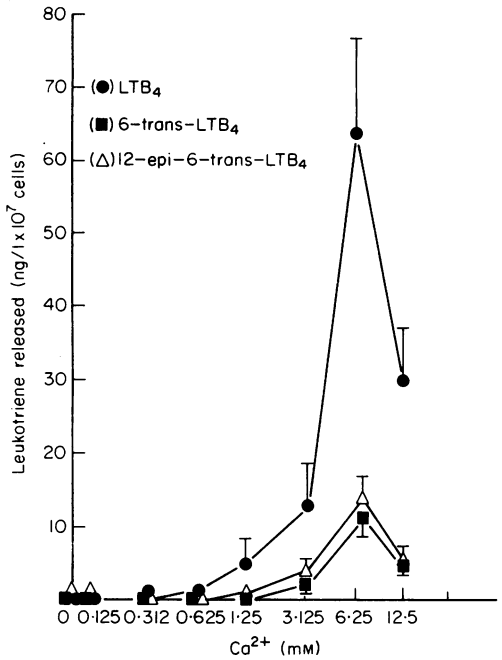

Figure 2. Calcium-dependent release of leukotrienes by stimulation of PMNs with opsonized zymosan. 1×10^7 PMNs were stimulated with 2 mg opsonized zymosan in the presence of different calcium concentrations for 30 min at 37°. Each value represents the mean ± SEM of four independent experiments with different donor cells.

Table 1. Magnesium-dependent leukotriene release on stimulation of PMNs with opsonized zymosan

Magnesium (mM)	Leukotriene release (ng/1 x 10^7 PMNs)		
	LTB_4	6-trans-LTB_4	12-epi-6-trans-LTB_4
0	6·65 ± 1·07	2·0 ± 0·4	2·0 ± 0·5
0·063	12·40 ± 1·80	2·1 ± 0·03	1·9 ± 0·3
0·160	11·70 ± 2·70	2·1 ± 0·03	2·7 ± 0·1
0·313	11·74 ± 1·85	1·7 ± 0·2	1·8 ± 0·3
0·625	11·31 ± 2·50	1·9 ± 0·5	1·4 ± 0·6
1·560	11·90 ± 2·30	2·5 ± 0·3	2·2 ± 0·5
3·125	11·00 ± 1·50	2·7 ± 0·8	1·5 ± 0·3
6·250	10·00 ± 2·90	2·1 ± 0·3	1·5 ± 0·4
12·500	4·30 ± 1·00	0	0

PMNs were incubated with opsonized zymosan (2 mg/ 1×10^7 cells) for 30 min in the presence of 1·25 mM calcium and different concentrations of magnesium: analysis of leukotriene release by HPLC. Each value represents the mean ± SEM of four independent experiments with different donor cells.

concentration of 12·5 mM Ca^{2+} only 30 ± 7·9 ng $LTB_4/1 \times 10^7$ cells were detected ($n=4$)]. Optimal amounts of LTC_4 release were obtained at calcium concentrations ranging from 3·15 to 12·5 mM (data not shown). At a constant concentration of calcium (1·25 mM), the addition of magnesium up to 0·063 mM caused an increase in LTB_4 release from PMNs stimulated with opsonized zymosan (Table 1). The amounts of LTB_4 ranged from 6·65 ± 1·07 ng/1 x 10^7 cells ($n=4$) in the absence of magnesium to 11–12 ng in the presence of magnesium. Magnesium concentrations above 6 mM led to a decrease in LTB_4 release. The release of 6-trans-LTB_4, 12-epi-6-trans-LTB_4 (Table 1) and LTC_4 was not affected significantly at any concentration of magnesium. In contrast, when PMN were stimulated with Ca ionophore ($7{\cdot}3 \times 10^{-6}$ M), a calcium concentration above 2 mM led to a significant decrease in LTB_4 and LTC_4 release (Fig. 3).

Magnesium did not significantly change the leukotriene release from Ca ionophore-stimulated PMNs (data not shown). The stimulation of PMNs with opsonized zymosan under the described conditions led to the release of lysozyme (50–55% of

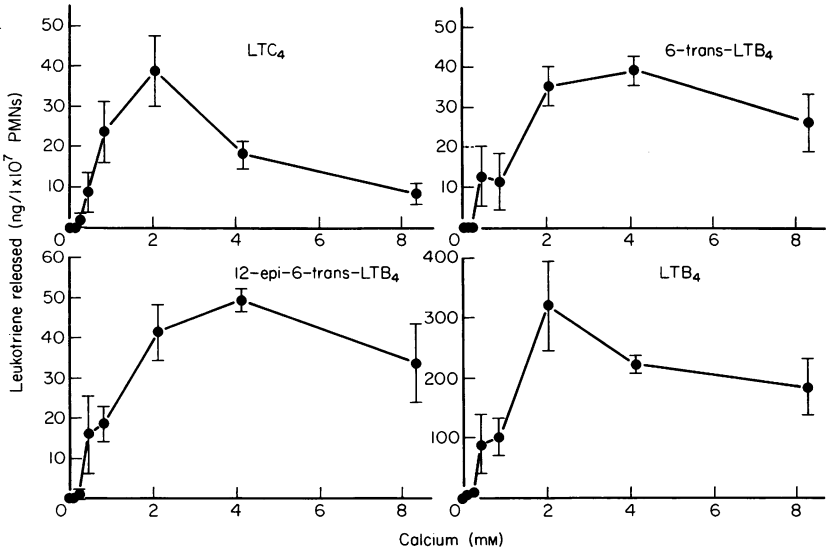

Figure 3. Calcium-dependent leukotriene release on stimulation of PMNs (1×10^7) with $7{\cdot}3 \times 10^{-6}$ M Ca ionophore. PMNs were stimulated in the presence of different calcium concentrations for 15 min at 37°. Leukotriene release was measured by HPLC. Each value represents the mean ± SEM of three different experiments.

Table 2. Metabolism of exogenously added LTC_4 by stimulated PMNs and percentage distribution of the metabolites

Sample	Time of prestimulation (min)	Exogenous LTC_4 (%)	Generated		% LTC_4 metabolized
			6-trans-LTB_4 (%)	12-epi-6-trans-LTB_4 and LTD_4 (%)	
Cells + Ca^{2+}/Mg^{2+} + Zx*	0	57	19	24	43
Ca^{2+}/Mg^{2+}	3	26	36	38	74
Ca^{2+}/Mg^{2+}	6	27	33	41	73
Ca^{2+}/Mg^{2+}	10	15	37	48	85
Ca^{2+}/Mg^{2+}	20	33	28	39	67
Ca^{2+}/Mg^{2+}	30	43	25	32	57
Ca^{2+}/Mg^{2+}	60	56	19	25	44
Cells + PBS + Zx	60	94	0	6	6
Cells + Ca^{2+}/Mg^{2+} + PBS	60	95	0	5	5
Cells + PBS + PBS	60	100	0	0	0

1×10^7 PMNs were preincubated with opsonized zymosan in the presence of calcium and magnesium for different times at 37°. After prestimulation, 150 ng LTC_4 were added to the complete cell suspension and the incubation was continued for further 15 min. The metabolites were analysed by HPLC. Data are expressed as the percentage of identified leukotriene areas. The amount of the whole identified leukotrienes is expressed as 100%. The data represent the metabolite distribution of a typical experiment; the donor-specific variation of the LTC_4 metabolism was calculated as $\pm 15\%$ and was derived from $n = 3$ experiments.

* Zx = opsonized zymosan.

total enzyme content), whereas only small quantities of β-glucuronidase (5–10% of total) were detected. The release of lactate dehydrogenase (LDH) was always less than 5%, indicating that the cells remained intact during the incubation and stimulation (data not shown).

In the past, we have presented evidence to suggest that stimulation of human PMNs with the Ca ionophore and opsonized zymosan led to the release of leukotriene-transforming enzymes, such as γ-glutamyl transpeptidase (LTC_4 conversion to LTD_4) and LTD_4-dipeptidase (LTD_4 conversion to LTE_4) into the cell-free supernatant (Raulf *et al.*, 1985). When leukotriene C_4 was added to the unseparated cell suspension (1×10^7 PMN) stimulated with opsonized zymosan (2 mg) for various times in the presence of Ca^{2+} (1·25 mM) and Mg^{2+} (3·125 mM), a type of leukotriene metabolism differing from the one previously described was observed (Table 2). Exogenously added LTC_4 was metabolized into 6-trans-LTB_4, 12-epi-6-trans-LTB_4 and LTD_4 (cochromatography of LTD_4 and 12-epi-6-trans-LTB_4; see the Materials and Methods). Our HPLC system was not suitable for the detection of metabolites that behaved in a way more polar than LTC_4, e.g. the sulphoxides (Lee *et al.*, 1983). The formation of these metabolites paralleled the decrease in the amount of exogenously added LTC_4. Table 2 further demonstrates that almost complete metabolism (70–85%) of the added LTC_4 was already detectable when the cells were preincubated for 3–10 min with opsonized zymosan in the presence of Ca^{2+} and Mg^{2+}. After 60 min of preincubation, only 56% of the added LTC_4 was detected while the remainder was metabolized. However, in the absence of calcium and magnesium (cells + PBS + Zx) the rate of LTC_4 metabolism decreased to 6%, with prestimulated cells analysed after 60 min. Insignificant metabolism was obtained for the controls (e.g. cells in the presence of cations and cells in the absence of cations suspended in PBS).

Experiments were then carried out to analyse the kinetics of LTC_4 metabolism. Human PMNs (1×10^7) were prestimulated with opsonized zymosan for 15 min at 37° in the presence of Ca^{2+} and Mg^{2+}, LTC_4 was added and the incubation was continued. After 2 min of incubation, $34{\cdot}3 \pm 13{\cdot}6\%$ ($n = 3$) of the exogenous LTC_4 was metabolized to LTB_4 isomers. After 20 min of incubation more than 70% of the exogenous LTC_4 was transformed into LTB_4 isomers.

The results obtained by HPLC analysis were further supported by experiments using radiolabelled leukotrienes by studying the distribution of the generated metabolites by thin-layer chromatography. The incubation of opsonized zymosan prestimulated PMNs with [^{3}H]LTC_4 for a further 15 min confirmed the data obtained with unlabelled LTC_4. Depending on the preincubation time of the cells with opsonized zymosan in the presence of Ca^{2+} and Mg^{2+}, the amount of [^{3}H]LTC_4 decreased, and metabolites corresponding to LTB_4 and its isomers ($R_f = 0{\cdot}90 \pm 0{\cdot}01$) and a metabolite that behaved in a way more polar than LTC_4 ($R_f = 0{\cdot}19 \pm 0{\cdot}01$) were generated (as was pointed out in the Materials and Methods, TLC does not distinguish between LTB_4 and LTB_4 isomers). Optimal metabolism of [^{3}H]LTC_4 was detectable after 5–15 min of prestimulating the cells. After longer times of prestimulation (30–60 min), less [^{3}H]LTC_4 (50% versus 77–83% after 5–15 min) was metabolized into LTB_4 isomers.

It has been suggested that the products of the oxidative burst might be involved in the leukotriene metabolism (Henderson & Klebanoff, 1983b; Lee *et al.*, 1982, 1983). Therefore, experiments were performed by adding inhibitors of the oxidative burst to the cell suspension which was prestimulated for 15 min with opsonized zymosan in the presence of calcium and magnesium (Table 3). According to Lee *et al.* (1982) and Henderson & Klebanoff (1983b), the addition of 12 mM L-serine, sodium azide (1·2 mM) or catalase (30 μg) inhibited the LTC_4

metabolism to LTB_4 isomers significantly; superoxide dismutase (3·1 μg), however, did not affect the metabolism.

Experiments were then performed to analyse the effect of stimuli other than opsonized zymosan with regard to the LTC_4 turnover. For this purpose, cells were prestimulated for 15 min in the presence of calcium and magnesium with opsonized zymosan, fMLP, Ca ionophore or PMA (Table 4). It became apparent that exogenously added [^{3}H]LTC_4 was metabolized to a different degree. Only minute LTC_4 metabolism to LTB_4 isomers was detected when cells were stimulated with fMLP (2×10^{-4} M) or low concentrations of Ca ionophore ($1{\cdot}6$–$3{\cdot}2 \times 10^{-6}$ M). With increasing concentrations of Ca ionophore ($7{\cdot}9$–16×10^{-6} M), the amount of metabolized LTC_4 increased up to $22 \pm 4\%$ ($n=3$). When PMA ($2{\cdot}5 \times 10^{-6}$ M) or opsonized zymosan (2 mg) was analysed, either 73% ($n=2$) or $83{\cdot}5 \pm 4{\cdot}8\%$ ($n=3$) of the added LTC_4 was transformed into LTB_4 isomers.

Transformation of [^{3}H]LTD_4 (Table 5) by cell suspensions prestimulated with opsonized zymosan in the presence of calcium and magnesium also led to metabolites corresponding to LTB_4 and LTB_4 isomers. However, the time-range of prestimulation had lesser effects on the rate of LTD_4 metabolism as compared with LTC_4 metabolism. Cell suspensions obtained after 60 min of prestimulation with opsonized zymosan in the presence of 1·25 mM calcium and 3·125 mM magnesium were able to metabolize $38 \pm 4\%$ ($n=3$) of the exogenously added [^{3}H]LTD_4 to LTB_4 isomers. Similar results were already obtained after 5 min of prestimulation. When cells were

Table 3. Effects of inhibitors of the oxidative burst on [^{3}H]LTC_4 metabolism

Inhibitors	% [^{3}H]LTC_4 metabolized	% inhibition
None	$83{\cdot}5 \pm 4{\cdot}8$	0
L-serine (112 mM)	$47{\cdot}5 \pm 2{\cdot}5$	43
Catalase (30 μg)	$40{\cdot}0 \pm 2{\cdot}0$	50
Sodium azide (1·2 mM)	$56{\cdot}5 \pm 5{\cdot}5$	32
SOD (3·1 μg)	$85{\cdot}0 \pm 6{\cdot}0$	0

PMNs were prestimulated for 15 min with opsonized zymosan and calcium (1·25 mM) and magnesium (3·125 mM) in the presence of the inhibitors or with the same volume of buffer (0% inhibition), [^{3}H]LTC_4 (0·1 μCi) was added and the incubation was continued for 15 min. The amount of whole detectable and identified radioactivity was expressed as 100%. Each value represents the mean ± SEM of three independent experiments with different donor cells.

Table 4. Effects of different stimuli on [^{3}H]LTC_4 metabolism

Stimuli	Concentration	% [^{3}H]LTC_4 metabolized
Opsonized zysosan	2 mg	$83{\cdot}5 \pm 4{\cdot}8$
fMLP	2×10^{-4} M	$3{\cdot}3 \pm 3{\cdot}3$
Ca ionophore	$1{\cdot}6 \times 10^{-5}$ M	$22{\cdot}0 \pm 4{\cdot}0$
Ca ionophore	$7{\cdot}9 \times 10^{-6}$ M	$22{\cdot}0 \pm 15{\cdot}6$
Ca ionophore	$3{\cdot}2 \times 10^{-6}$ M	$5{\cdot}3 \pm 5{\cdot}3$
Ca ionophore	$1{\cdot}6 \times 10^{-6}$ M	0
PMA	$2{\cdot}5 \times 10^{-6}$ M	73·0*

PMNs were prestimulated in the presence of calcium (1·25 mM) and magnesium (3·125 mM) for 15 min with different stimuli; [^{3}H]LTC_4 (0·1 μCi) was added and the incubation was continued for 15 min. The amount of whole detectable and identified radioactivity was expressed as 100%. Each value represents the mean ± SEM of three independent experiments.

* Mean of two determinations.

Table 5. Metabolism of [^{3}H]LTD_4 (0·1 μCi) in cell suspensions prestimulated with opsonized zymosan for various times

Sample	Time of prestimulation (min)	% [^{3}H]LTD_4 metabolized: to [^{3}H]LTB_4 and isomers (R_f = p·90 + 0·01)	to [^{3}H]LTE_4 ($R_f = 0{\cdot}73 \pm 0{\cdot}02$)
Cells + Ca^{2+}/Mg^{2+} + Zx*	0	$26{\cdot}0 \pm 7{\cdot}0$	0
Ca^{2+}/Mg^{2+}	5	$39{\cdot}0 \pm 3{\cdot}5$	0
Ca^{2+}/Mg^{2+}	15	$42{\cdot}0 \pm 3{\cdot}0$	0
Ca^{2+}/Mg^{2+}	30	$35{\cdot}0 \pm 3{\cdot}0$	0
Ca^{2+}/Mg^{2+}	60	$38{\cdot}0 \pm 4{\cdot}0$	0
Cells + Ca^{2+}/Mg^{2+} + PBS	60	0	$76{\cdot}0 \pm 17{\cdot}0$
Cells + PBS + Zx	60	$5{\cdot}3 \pm 5{\cdot}3$	$70{\cdot}0 \pm 18{\cdot}0$
Cells + PBS + PBS	60	0	$58{\cdot}0 \pm 9{\cdot}0$

Subsequently the cell suspension was incubated with [^{3}H]LTD_4 for a further 15 min (time-point 0 min = incubation of cells with opsonized zymosan and [^{3}H]LTD_4 simultaneously for 15 min; time-point 5 min = incubation of cells with opsonized zymosan for 5 min, subsequent addition of [^{3}H]LTD_4 and further incubation for 15 min). The radioactivity of the identified metabolites was expressed as 100%. Each value represents the mean ± SEM of three independent experiments.

* Zx = opsonized zymosan.

stimulated with opsonized zymosan in the presence of [^{3}H]LTD$_4$ for 15 min (time-point 0 min), 26·0 ± 7·0% (n = 3) was transformed into LTB$_4$ isomers. Cells stimulated in the absence of calcium and magnesium with opsonized zymosan for 60 min metabolized [^{3}H]LTD$_4$ by 70% to LTE$_4$ and only small amounts (5·3 ± 5·3% n = 3) to LTB$_4$ isomers. Cell suspension incubated with PBS for 60 min in the presence and absence of cations metabolized 76 ± 17% and 58 ± 9% (n = 3) of the added [^{3}H]LTD$_4$ to LTE$_4$ after a subsequent incubation for 15 min.

DISCUSSION

Our data demonstrate that stimulation of human polymorphonuclear granulocytes with opsonized zymosan led to a time-dependent release of LTB$_4$ with a maximum after 20 min of incubation. A similar pattern of LTB$_4$ release was described by Claesson, Lundberg & Malmsten (1981). They showed a plateau phase after 10–20 min of incubation. The amount of LTB$_4$ released by stimulation with opsonized zymosan only comprised a small fraction (15%) of that as obtained with the Ca ionophore. Stimulation of PMN with fMLP also led to minor LTB$_4$ release as compared to the above-mentioned stimuli. Similar observations were described by Palmer & Salmon (1983). With regard to LTC$_4$, our results show that with opsonized zymosan only a small amount of LTC$_4$ was detectable in the supernatant as compared to stimulation with the Ca ionophore. One has to consider that the amounts of leukotrienes detectable reflect the net amounts resulting from *de novo* synthesis and metabolism. An interpretation of the quantitative differences in leukotriene formation and release after Ca ionophore and zymosan stimulation of PMN was suggested by Williams *et al.* (1985). They demonstrated that, in contrast to the Ca ionophore, stimulation of PMNs with unopsonized zymosan resulted in a retention of 5-lipoxygenase products within the cells. Thus, release of these metabolites may be an event that is regulated separately from their generation.

The leukotriene release by stimulation with opsonized zymosan was completely dependent on the presence of divalent cations. Only calcium concentrations higher than 1·25 mM led to LTB$_4$ release that could be detected by HPLC analysis. Optimal LTB$_4$ release was obtained at a calcium concentration of 6·25 mM. In the presence of magnesium, yet at a constant calcium concentration, leukotriene release was enhanced. Leukotriene release on stimulation with the Ca ionophore was detectable at a calcium concentration of 0·09 mM and reached its optimum at concentrations ranging from 0·9 to 1·8 mM calcium. The induction of leukotriene release by Ca ionophore was not affected by magnesium. Enhancement of leukotriene release in the presence of high concentrations of divalent cations suggested that cations are required for leukotriene release. The importance of the divalent cations calcium and magnesium for the stimulation of the oxidative metabolism in PMNs with opsonized zymosan (measured by different chemiluminescence techniques) was described by Williams & Cole (1981). They showed that the binding of PMN to opsonized zymosan requires magnesium, while uptake requires both calcium and magnesium. Our data demonstrated that the induction of leukotriene release by opsonized zymosan was affected by calcium rather than magnesium. The regulatory role of divalent cations is a complex field to study since both influxes and effluxes have to be considered (Hoffstein, 1980). The present analysis was directed to optimize the incubation conditions for the release of leukotrienes *in vitro*.

We previously demonstrated that stimulation of PMNs with opsonized zymosan and Ca ionophore also led to the release of leukotriene-transforming activity such as γ-glutamyl- transpeptidase and LTD$_4$ dipeptidase into the cell supernatants (Raulf *et al.*, 1985).

The experiments described here suggest that stimulation of neutrophils with opsonized zymosan and Ca ionophore as well as PMA (Lee *et al.*, 1982; Henderson *et al.*, 1983b) led to another pathway of metabolism when the synthetic leukotrienes C$_4$ or D$_4$ were added to the unseparated cell suspension. In this case, the principal metabolites of LTC$_4$ were the LTB$_4$ isomers 6-trans-LTB$_4$ and 12-epi-6-trans-LTB$_4$, which cochromatographed with LTD$_4$ by HPLC. Studies investigating the metabolism of [^{3}H]LTC$_4$ with subsequent TLC analysis revealed a metabolite more polar than LTC$_4$, and a metabolite corresponding to LTB$_4$ and its isomers; however, no LTD$_4$ was detected. Thus, there is evidence to suggest that the pathway of oxidative LTC$_4$ metabolism leads to the two LTB$_4$ isomers (6-trans-LTB$_4$ and 12-epi-6-trans-LTB$_4$) as main products and to a minor metabolite that behaves in a way more polar than LTC$_4$. Goetzl (1982) and Lee *et al.* (1982) also described the two LTB$_4$ isomers 12 (R) and 12(S)-diastereoisomers of 6-trans-LTB$_4$ as metabolites of LTC$_4$ or LTD$_4$. Lee *et al.* (1982, 1983) detected polar products identified as two diastereoisomeric sulphoxides of LTC$_4$ or LTD$_4$, respectively. Our experiments clearly demonstrate that the rate of LTC$_4$ metabolism proved to be strongly dependent on the time of prestimulation with opsonized zymosan. The time of prestimulation apparently represents a defined cell-activation stage. The maximum of LTC$_4$ metabolism to LTB$_4$ isomers was observed after 3–15 min of prestimulation in the presence of calcium and magnesium. The metabolism of exogenously added LTC$_4$ to LTB$_4$ isomers was a fast process: more than 60% of the added LTC$_4$ was transformed after 5 min. Stimulation of neutrophil granulocytes by opsonized zymosan not only leads to the release of leukotrienes, but also causes the activation of the respiratory burst (Markert, Allaz & Frei, 1980), with increased consumption of oxygen and the production of reactive substances including the superoxide anion, hydrogen peroxide and hydroxyl radicals. The stimulation of the myeloperoxidase–H_2O_2–halide system (HOCl generation) (Lee *et al.*, 1983) as well as the release of OH· radicals (Henderson *et al.*, 1983a) could play an important role in the inactivation of leukotrienes. The pathway of inactivation depends on the enzyme equipment of the cells studied (Henderson *et al.*, 1983b). Our experiments revealed that the addition of L-serine (Weller *et al.*, 1983), sodium azide and catalase (Henderson *et al.*, 1983b) significantly inhibited the metabolism of the exogenously added LTC$_4$ to LTB$_4$ isomers. In contast, SOD, which catalyses the conversion of superoxide anion to H_2O_2 and O_2, had no effect on this metabolism. These data indicated that superoxide anions are not involved in the oxidative LTC$_4$ metabolism; H_2O_2 formation (converted by catalase to H_2O and O_2) and the action of myeloperoxidase (inhibited by azide) are necessary for LTC$_4$ metabolism to LTB$_4$ isomers. The inhibitory effect of L-serine (HOCl scavenger) suggests that hypochlorous ions are also involved.

In contrast to leukotriene release, a comparison of the different stimuli showed that opsonized zymosan and PMA were more potent to activate the LTC$_4$ metabolism to LTB$_4$

isomers as compared to Ca ionophere. Prestimulation of granulocytes with fMLP results in minute or no conversion of exogenously added LTC_4 to LTB_4 isomers. The possible reason for this phenomenon, also described for human eosinophils by Goetzl (1982), could be the inactivation of fMLP by myeloperoxidase (Lane & Lamkin, 1983). In a recent publication (Neill, Henderson & Klebanoff, 1985), it has been suggested that LTC_4 degradation to the LTB_4 isomers by normal human monocytes is also stimulus-dependent. In this report too, PMA and opsonized zymosan are more potent to activate the LTC_4 degradation as compared to Ca ionophore or fMLP.

The metabolism of $[^3H]LTD_4$ to $[^3H]LTB_4$ isomers was dependent on the presence of calcium and magnesium and opsonized zymosan, respectively. In the absence of one or both of these components, prestimulated cells metabolized exogenously added LTD_4 via a LTD_4 dipeptidase to LTE_4.

Thus, our data support the notion that two ways exist for the metabolism of the peptido-leukotrienes LTC_4 and LTD_4 by neutrophil granulocytes during stimulation: (i) the secretion of the leukotriene-transforming enzymes γ-glutamyl-transpeptidase and LTD_4-dipeptidase finally results in the formation of LTE_4, and, as a consequence, the spasmogenic activity of the cell supernatants is reduced, and (ii) the secretion of metabolites of the respiratory burst leads to an inactivation of the peptido-leukotrienes LTC_4 and LTD_4 into LTB_4 isomers; the spasmogenic activity is almost lost completely. Further study is needed to investigate whether the generation of LTB_4 isomers also reduces the influx of phagocytes, thus counteracting the inflammatory response by deactivating the cells.

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