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The leukotriene B_4 paradox: neutrophils can, but will not, respond to ligand–receptor interactions by forming leukotriene B_4 or its ω-metabolites

Kathleen A. HAINES,*†‡ Kenneth N. GIEDD,* Abby M. RICH,* Helen M. KORCHAK* and Gerald WEISSMANN*

*Department of Medicine, Division of Rheumatology and †Department of Pediatrics, NYU Medical Center, New York, NY 10016, U.S.A.

Leukotriene B_4 (5*S*,12*R*-dihydroxy-6,14-*cis*, 8,10-*trans*-eicosatetraenoic acid, LTB_4) is released from neutrophils exposed to calcium ionophores. To determine whether LTB_4 might be produced by ligand–receptor interactions at the plasmalemma, we treated human neutrophils with serum-treated zymosan (STZ), heat-aggregated IgG and fMet-Leu-Phe (fMLP), agonists at the C3b, Fc and fMLP receptors respectively. STZ (10 mg/ml) provoked the formation of barely detectable amounts of LTB_4 (0.74 ng/10^7 cells); no ω-oxidized metabolites of LTB_4 were found. Adding 10 μM-arachidonate did not significantly increase production of LTB_4 or its metabolites. Addition of 50 μM-arachidonate (an amount which activates protein kinase C) before STZ caused a 40-fold increase in the quantity of LTB_4 and its ω-oxidation products. Neither phorbol myristate acetate (PMA, 200 ng/ml) nor linoleic acid (50 μM), also activators of protein kinase C, augmented generation of LTB_4 by cells stimulated with STZ. Neither fMLP (10^{-6} M) nor aggregated IgG (0.3 mg/ml) induced LTB_4 formation (<0.01 ng/10^7 cells). Moreover, cells exposed to STZ, fMLP, or IgG did not form all-*trans*-LTB_4 or 5-hydroxyeicosatetraenoic acid; their failure to make LTB_4 was therefore due to inactivity of neutrophil 5-lipoxygenase. However, adding 50 μM-arachidonate to neutrophil suspensions before fMLP or IgG triggered LTB_4 production, the majority of which was metabolized to its ω-oxidized products (fMLP, 20.2 ng/10^7 cells; IgG, 17.1 ng/10^7 cells). The data show that neutrophils exposed to agonists at defined cell-surface receptors produce significant quantities of LTB_4 only when treated with non-physiological concentrations of arachidonate.

INTRODUCTION

Leukotriene B_4 (LTB_4) is among the most potent inflammatory mediators generated by human cells [1]. LTB_4 causes neutrophils to move to a site of inflammation, degranulate and generate active oxygen species [2–5]. Neutrophils themselves are capable of synthesizing large quantities of LTB_4 when stimulated with calcium ionophore in the absence of exogenous arachidonic acid [6]. It has been postulated, therefore, that LTB_4 is a necessary intermediate in the acute inflammatory response [7–10]. To define the conditions under which neutrophils release LTB_4, we have studied synthesis of LTB_4 in response to agonists at receptors which mediate phagocytosis, enzyme secretion and chemoattraction: (1) the C3b component of complement (serum-treated zymosan, STZ), (2) the Fc portion of IgG (heat-aggregated IgG), and (3) the synthetic chemoattractant fMet-Leu-Phe (fMLP). We show that LTB_4 is synthesized in barely detectable quantities by neutrophils stimulated with these ligands. It is produced in significant amounts only when cells are loaded with arachidonic acid in quantities (50 μM) that stimulate $O_2^{-\cdot}$ production and activate protein kinase C (PKC) [11,12]. Lower concentrations of arachidonate (10 μM) did not augment the STZ-stimulated synthesis of LTB_4. We further show that activation of PKC is not a sufficient co-signal for generation of LTB_4 since phorbol myristate acetate (PMA) and linoleic acid (50 μM) both of which stimulate $O_2^{-\cdot}$ production and activate PKC [11,12], do not act synergistically with other ligands to stimulate LTB_4 synthesis. These studies pose a paradox: a potent mediator of inflammation produced by neutrophils seems not to be released in response to agonists of receptors which provoke phagocytosis, degranulation and chemotaxis.

MATERIALS AND METHODS

Buffers and reagents

Hepes buffer was 150 mM-Na^+, 5 mM-K^+, 1.2 mM-Mg^{2+}, 1.3 mM-Ca^{2+}, 155 mM-Cl^-, 10 mM-Hepes and 5 mM-glucose, pH 7.45; phosphate-buffered saline (PBS) contained 0.9% NaCl, 0.01 M-PO_4^{2-} and 5 mM-glucose, pH 7.42; ACD was 67 mM-citric acid, 85 mM-sodium citrate and 110 mM-glucose. Cytochalasin B was from Aldrich, arachidonic acid was from Nu Chek, and

Abbreviations used: fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; LTB_4, leukotriene B_4 (5*S*,12*R*-dihydroxy-6,14-*cis*, 8,10-*trans*-eicosatetraenoic acid; all-*trans* LTB_4, 5*S*,12*S*-dihydroxy-6,8,10-*trans*, 14-*cis*-eicosatetraenoic acid and 5*S*,12*R*-dihydroxy-6,8,10-*trans*-, 14-*cis*-eicosatetraenoic acid; 5*S*-monoHETE, 5*S*-hydroxy-6,10-*trans*, 8,14-*cis*-eicosatetraenoic acid; 5*S*,12*S*-diHETE, 5*S*,12*S*-dihydroxy-6,10-*trans*, 8,14-*cis*-eicosatetraenoic acid; ω-oxidized LTB_4, 5*S*,12*R*-20-trihydroxy-6,14-*cis*, 8,10-*trans*-eicosatetraenoic acid and 5*S*,12*R*-dihydroxy-20-carboxy-6,14-*cis*, 8,10-*trans*-eicosatetraenoic acid; PMA, phorbol myristate acetate; PKC, protein kinase C; $O_2^{-\cdot}$, superoxide anion.

‡ To whom correspondence and reprint requests should be addressed.

A23187 was from Calbiochem; methanol, hexane, propan-2-ol and acetonitrile were h.p.l.c. grade from Burdick and Jackson; anhydrous diethyl ether, h.p.l.c. grade, was from J. T. Baker. Standard LTB_4, ω-oxidized LTB_4, all-*trans*-LTB_4 isomers and mono-HETEs were kind gifts from Dr S. Shak, NYU Medical Center, Dr P. Borgeat, University of Laval, Quebec, Canada, and Dr J. Rokach, Merck Frosst, Canada, and Dr C. N. Serhan, Karolinska Institute, Stockholm, Sweden.

Cell separation

Pools of human polymorphonuclear leukocytes from normal adult volunteers were isolated by centrifugation over a Ficoll–Hypaque cushion and subsequent dextran sedimentation according to the method of Boyum [13]. Remaining erythrocytes were removed by hypo-osmotic lysis.

Extraction and separation procedures

Lipoxygenase products were isolated by ether extraction according to the method of Borgeat & Samuelsson [14,15]. Briefly, the stimulated cell mixture was precipated with 2 vol. of methanol to extract both intra- and extra-cellular LTB_4 and kept overnight at -20 °C. It was then centrifuged at 1600 *g* at 4 °C in a Sorval RC3 centrifuge, the supernatant was decanted, and the pH was adjusted to 3.0. The supernatant was then extracted with ether using an ether/water/supernatant mixture of 2:1:1 (by vol.). The resulting water phase was discarded, and the ether phase was washed twice more with water. The ether phase was blown to dryness under a stream of N_2. Alternatively, the arachidonic acid metabolites were purified according to the method of Powell [16]. The supernatant was diluted to a concentration of 15% methanol in water and applied to a Sep-Pak C_{18} cartridge which was then washed with water (20 ml) and light petroleum (20 ml) sequentially. The lipoxygenase products were eluted from the column with methanol or methyl formate, which was evaporated under a stream of N_2. The arachidonate metabolites were then separated on an Altex Ultrasphere ODS (5 μm; 4.6 mm × 25 cm) reverse-phase h.p.l.c. column (Beckman Instruments) using a solvent system of methanol/water/acetic acid (75:25:0.01, by vol.). The eluant was monitored at 270 nm for diHETEs and 235 nm for monoHETEs. Fractions were collected, dried under a stream of N_2 and resuspended in methanol until further use. Concentrations of products were determined by measuring u.v. absorbance, at 270 nm for diHETEs using an absorption coefficient of 45000 $M^{-1} \cdot cm^{-1}$, and at 235 nm for mono-HETEs using an absorption coefficient of 30500 $M^{-1} \cdot cm^{-1}$. Average recovery of arachidonic acid metabolites were determined by comparison of the recovery of 100 ng of prostaglandin B_2 which was added to the reaction mixture, as measured by the area under its h.p.l.c. elution peak. The average for six experiments was 105% ±30% (S.E.M.). Recovery of LTB_4 was determined by addition of [^{3}H]LTB_4 to cell suspensions and determining [^{3}H]LTB_4 and ω-oxidized [^{3}H]LTB_4, which was 73% of that added.

Purity of LTB_4

Contamination of LTB_4 with the double lipoxygenase product was determined according to the method of Borgeat *et al.* [17] as previously reported [18]. The quantity of 5*S*,12*S*-diHETE never exceeded 5%.

Identification of ω-oxidized metabolites

ω-Oxidized metabolites were further purified according to the method of Shak & Goldstein [19]. The void volume from the initial h.p.l.c. separation was collected, applied to the reverse phase column and eluted with a step gradient system of methanol/acetonitrile/water/acetic acid (35:25:45:0.02, by vol.) to a final solution of methanol/acetonitrile (75:25, v/v).

Neutrophil aggregation

Aggregation was measured as previously described by Craddock *et al.* [20]. Briefly, 1.25×10^6 cells were stirred in a 100 μl silicone-treated cuvette in Hepes buffer containing 5 μg of cytochalasin B/ml. They were stimulated with various concentrations of LTB_4. Aggregation was measured as an increase in light transmission with respect to time.

Superoxide anion generations

$O_2^{-\cdot}$ generation was measured by the superoxide dismutase inhibitable reduction of cytochrome *c* as reported previously [21].

Zymosan preparation

Zymosan (ICN Pharmaceuticals) was suspended in water, boiled for 30 min and washed twice in PBS. The pelleted particles were suspended in freshly prepared serum at a concentration of 10 mg/ml in incubated for 30 min at 37 °C on a rotating wheel. The particles were washed twice in Hepes buffer and used within 1 h.

Cell stimulation

Cells were suspended at a concentration of 35×10^6/ml and incubated for 15 min at 37 °C with the appropriate amount of arachidonic acid or linoleic acid in ethanol. The ethanol concentration never exceeded 0.1% by volume. Where appropriate, the cells were added to pelleted STZ which was then resuspended to achieve a final concentration of 10 mg of STZ/ml of cell suspension. The reaction was allowed to proceed for 10 min where, upon examination, > 90% of the cells had ingested three or more particles. It was then stopped by the addition of 2 vol. of methanol. Alternatively, fMLP in Hepes buffer, IgG in Hepes buffer, PMA in Hepes buffer or A23187 in dimethyl sulphoxide were added to achieve the desired concentrations, and the reaction was again stopped after 10 min. Doses of stimuli were chosen to fall within the secretory ranges for the various stimuli [21–24]. The presence of 50 μM-arachidonate did not inhibit enzyme secretion provoked by these stimuli.

Measurements of cytosolic Ca^{2+}

Fura-2 AM was purchased from Molecular Probes Inc. (Junction City, OR, U.S.A.). Neutrophils were suspended at 10^8/ml in 10^{-5} M-Fura-2 AM in Hepes buffer, pH 7.4, at 37 °C for 5 min. They were then diluted 10-fold with Hepes buffer and further incubated for 20 min at 37 °C. They were washed twice. Preloaded cells (2 ml, 5×10^6 cells/ml) were placed in a 1 cm^2 quartz cuvette heated to 37 °C. Fluorescence changes were monitored without stirring at 37 °C with an excitation wavelength of 340 and 380 nm and emission wavelength of 510 nm in a Perkin–Elmer 650–10S spectrofluorimeter. Levels of intracellular Ca^{2+} were calculated according to the equations of Grynkiewicz *et al.* [25].

RESULTS

Effect of STZ, FMLP and IgG on LTB_4 production

In order to determine whether serum-treated zymosan was capable of stimulating LTB_4 production, neutrophils were incubated with 10 mg of STZ/ml for 10 min at 37 °C. In contrast with the quantity of LTB_4 produced by cells treated with 10 μM-A23187, neutrophils exposed to STZ produced only 0.74 ± 0.21 ng of $LTB_4/10^7$ cells (Table 1), a barely detectable quantity, identified only by its retention time on reverse-phase h.p.l.c. Treatment of neutrophils with fMLP (10^{-6} M) produced similar amounts of LTB_4 (Table 1). When added to cell suspensions in the presence of STZ, 10^{-6} M-fMLP failed to increase the amounts of LTB_4 produced (1.79 ± 0.5 ng/10^7 cells). Treatment of neutrophils with heat-aggregated IgG (0.3 mg/ml) provoked no detectable amount of LTB_4 synthesis (Table 1). Moreover, neutrophils exposed to STZ, fMLP, or IgG formed only minimal amounts of 5-HETE or all-*trans*-LTB_4s (Table 1), indicating that neutrophil 5-lipoxygenase was not activated under these conditions.

Effect of PMA on STZ-treated neutrophils

Liles *et al.* [26] have found that activation of PKC by PMA augments LTB_4 production by neutrophils exposed to low concentrations (0.4 μM) of the ionophore A23187. To determine whether PMA might act in synergy with another stimulus to provoke LTB_4 generation, we added PMA together with STZ to neutrophil suspensions. PMA did not cause neutrophils to generate LTB_4 (Table 1), nor did PMA act in synergy with STZ to induce further LTB_4 synthesis (Table 2).

Effect of exogenous arachidonate on LTB_4 generation

Marcus *et al.* [27] reported that neutrophils utilize exogenous arachidonic acid to produce lipoxygenase products when exposed to calcium ionophore. We have previously shown [18] that 150 μM-arachidonate added to neutrophils in the absence of other stimuli results in the production of monoHETEs but not of diHETEs. In order to determine whether neutrophils produce LTB_4 in response to inflammatory ligands only in the presence of exogenous arachidonic acid, we preincubated neutrophils for 15 min with 1 μM-, 10 μM- and 50 μM-arachidonate and exposed the cells to STZ. Neutrophils that had been preincubated for 15 min with 1 μM- or 10 μM-arachidonic acid made 0.62 ng and 1.18 ng/10^7 cells respectively. No ω-oxidation products were found (Table 2). This does not differ significantly from the quantity of LTB_4 resulting from STZ treatment without exogenous arachidonate. In contrast, 15.7 ng of $LTB_4/10^7$ cells was recovered from cell suspensions that had been preincubated with 50 μM-arachidonate before treatment with

Table 1. Effect of various stimuli on formation of 5-lipoxygenase products by neutrophils

Neutrophils were preincubated for 15 min at 37 °C and the appropriate stimulus was then added. The cells were incubated for 10 min with all stimuli except A23187, which was for 5 min, and the reaction was stopped by the addition of an equal volume of methanol (see the Materials and methods section). Results are expressed as ng/10^7 cells ± S.E.M.

	Product (ng/10^7 cells)			
Stimulus	5-HETE	LTB_4	all-*trans*-LTB_4	ω-Oxidized LTB_4
A23187 (10 μM) ($n = 3$)	41.2 ± 10.6	49.7 ± 3.2	26.9 ± 4.3	17.1 ± 3.1
STZ (10 mg/ml) ($n = 8$)	0.40 ± 0.13	0.74 ± 0.21	0.37 ± 0.14	0
fMLP (10^{-6} M) ($n = 4$)	0	0.01 ± 0.01	0.05 ± 0.05	0
IgG (0.3 mg/ml) ($n = 2$)	0	0	0	0
PMA (200 ng/ml) ($n = 2$)	0	0	0	0

Table 2. Effect of exogenous arachidonate, linoleate or PMA on LTB_4 synthesis

Neutrophils were preincubated with lipid for 15 min at 37 °C and the appropriate stimulus was then added. The cells were incubated for 10 min and the reactions were stopped by the addition of an equal volume of methanol (see the Materials and methods section). Results are expressed as ng/10^7 cells ± S.E.M.

	Product (ng/10^7 cells)			
Stimulus	LTB_4	all-*trans*-LTB_4	ω-Oxidized LTB_4	LTB_4 + ω-oxidized LTB_4
STZ + 200 ng PMA ($n = 3$)	1.35 ± 0.9	0.6 ± 0.5	0	1.35 ± 0.9
STZ + 1 μM-arachidonate ($n = 9$)	0.45 ± 0.15	0.10 ± 0.08	0	0.45 ± 0.15
STZ + 10 μM-arachidonate ($n = 3$)	1.19 ± 0.44	0.17 ± 0.17	0	1.19 ± 0.44
STZ + 50 μM-arachidonate ($n = 7$)	15.55 ± 3.2	4.9 ± 1.0	20.7 ± 3.9	36.3 ± 5.6
STZ + 50 μM-linoleate ($n = 2$)	2.0 (1.1–2.9)	0	0	2.0 (1.1–2.9)
fMLP + 50 μM-arachidonate ($n = 3$)	1.20 ± 0.39	0	20.2 ± 4.6	21.4 ± 4.8
IgG + 50 μM-arachidonate ($n = 2$)	0.42 (0–0.84)	0	17.1 (14.7–19.4)	17.5 (14.7–20.2)

STZ ($P < 0.0002$). In addition, 20.7 ng/10^7 cells of the ω-oxidized metabolites of LTB_4 were also recovered, resulting in a total of 34.1 ng of LTB_4 synthesized/10^7 cells. The compound identified as LTB_4 (Fig. 1) not only displayed identical retention time with reference samples of LTB_4 [18] but yielded the triple absorbance maxima in the u.v. characteristic of a conjugated triene, i.e. peaks at 280, 270 and 259 nm. Moreover, its biological activity with respect to neutrophil aggregation was $114 \pm 20\%$ (S.D.) of that of purified biologically-derived LTB_4 (2×10^{-7} M). We also examined LTB_4 production by neutrophils exposed to either fMLP or to heat-aggregated IgG in the presence of 50 μM-arachidonic acid. When arachidonate was present, both fMLP and aggregated IgG provoked formation of LTB_4 which was recovered principally as ω-oxidation products. Neutrophils exposed to PMA in the presence and absence of 50 μM-arachidonate did not synthesize any LTB_4 (Table 2).

Trivial reasons for our inability to detect LTB_4 in neutrophil suspensions treated with arachidonate (1 or 10 μM) and STZ include: (1) arachidonic acid at concentrations lower than 50 μM does not enter cells, (2) neutrophil suspensions treated with fMLP or STZ contain enzymes which further metabolize ω-oxidized LTB_4 to products other than known metabolites, and (3) STZ is toxic to neutrophils in the absence of arachidonate and therefore no lipoxygenase products are produced. Each of these possibilities was tested.

Arachidonate at 1 or 50 μM was taken up with equal efficiency by neutrophils as determined by adding 1 μCi of [^{3}H]arachidonate to unlabelled arachidonate during the initial incubation and counting an aliquot of washed neutrophils before stimulation. Of the [^{3}H]arachidonate added with 1 μM-arachidonate, $72 \pm 3\%$ (S.E.M.) was taken up by cells; of the [^{3}H]arachidonate added with 50 μM-arachidonate, $68 \pm 7\%$ (S.E.M.) was taken up ($n = 3$).

Neutrophils stimulated with STZ or fMLP metabolize LTB_4 to the 20-hydroxy and 20-carboxy oxidation products, without further degradation. Trace amounts (0.08 μCi) of ω-oxidized [^{3}H]LTB_4 were added to neutrophils with or without 50 μM-arachidonic acid and the suspensions were treated with STZ or fMLP (10^{-6} M). ω-Oxidized [^{3}H]LTB_4 was recovered as a single peak on reverse-phase h.p.l.c. from cell suspensions treated with STZ $\pm$ 50 μM-arachidonate (Figs. 1 and 2) or fMLP $\pm$ 50 μM-arachidonate (results not shown). This peak of radioactivity was further resolved by the method of Shak & Goldstein [19]. It precisely co-eluted with standard 20-hydroxy-LTB_4 and 20-carboxy-LTB_4 (Figs. 1 and 2, insets). Recovery of ω-oxidized [^{3}H]LTB_4 from cells treated with STZ or STZ + 50 μM-arachidonate was 54% versus 51% ($n = 2$). Recovery of ω-oxidized [^{3}H]LTB_4 from cells treated with fMLP or fMLP $\pm$ 50 μM-arachidonate was 56% versus 55% respectively ($n = 2$).

Neutrophils treated with STZ $\pm$ archidonate were equally viable. Cells exposed to STZ released $6.7 \pm 1.5\%$ of the cytoplasmic enzyme, lactate dehydrogenase, as compared with cells treated with STZ and 50 μM-arachidonate, which released $9.2 \pm 1.0\%$ (S.E.M., $n = 3$). Therefore, the difference in formation of $LTB_4 \pm$ archidonate cannot be accounted for by cell death.

Dose–response studies with fMLP

fMLP is an effective chemoattractant at 10^{-8} M, but less so at 10^{-6} M [28]. These actions are reflected by appropriate changes in cytosolic Ca^{2+}. In contrast, $O_2^{-\cdot}$ generation reaches plateau levels only at 10^{-6} M. We

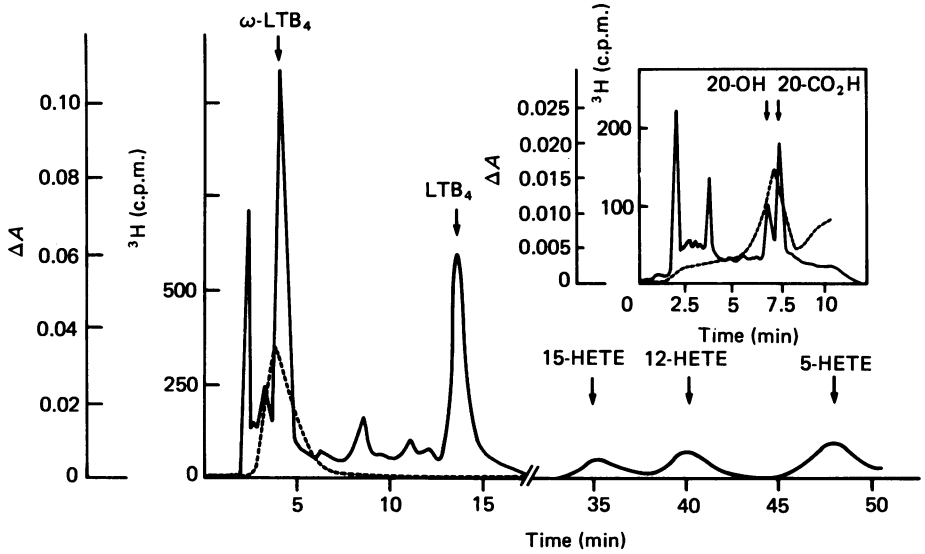

Fig. 1. Reverse-phase h.p.l.c. of neutrophils treated with STZ in the presence of 50 μM-arachidonic acid and ω-oxidized LTB_4

Neutrophils (3.5×10^7) were incubated for 15 min at 37 °C with 50 μM-arachidonic acid and 0.08 μCi of ω-oxidized [^{3}H]LTB_4, then treated for 10 min with STZ (10 mg/ml) at 37 °C. The reaction was stopped by the addition of 2 vol. of methanol. Extractions and reverse h.p.l.c. were performed according to the Materials and methods section. The column eluate was monitored at 270 nm for 25 min and at 235 nm for the subsequent 35 min. Fractions (1 ml) were collected and 100 μl aliquots were removed and monitored for radioactivity. Times of elutions of known standards are marked. Inset: the complex void volume was collected, pooled and subjected to repeat chromatography according to the Materials and methods section. The column eluate was monitored at 270 nm, collected in 1 ml fractions and 200 μl of each fraction was counted for the presence of radioactivity. Results are plotted as c.p.m.

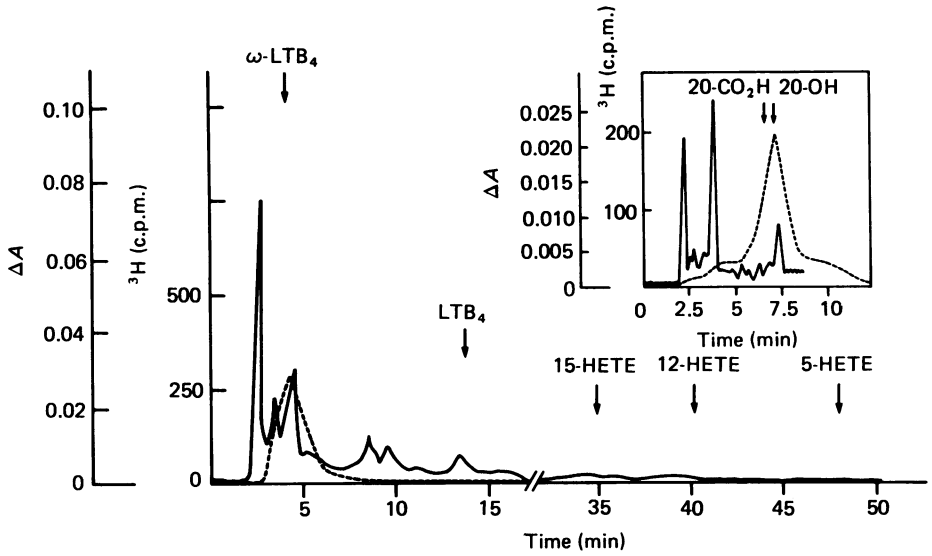

Fig. 2. Reverse-phase h.p.l.c. of neutrophils treated with STZ in the presence of ω-oxidized [^{3}H]LTB_4

Neutrophils (3.5×10^7) were incubated for 15 min at 37 °C with 0.08 μCi of ω-oxidized [^{3}H]LTB_4, then treated for 10 min with STZ (10 mg/ml) at 37 °C. The reaction was stopped by the addition of 2 vol. of methanol. Extractions and reverse-phase h.p.l.c. chromatography were performed according to the Material and methods section. Column eluate was monitored at 270 nm for 25 min and 235 nm for the subsequent 35 min. Fractions (1 ml) were collected and 100 μl aliquots were removed and monitored for radioactivity. Times of elutions of known standards are marked. Inset: the complex void volume was collected, pooled and subjected to repeat chromatography according to the Materials and methods section. The column eluate was monitored at 270 nm, collected in 1 ml fractions and 200 μl of each fraction was counted for the presence of radioactivity. Results are plotted as c.p.m.

therefore performed a dose–response analysis of LTB_4 secretion in response to fMLP. At no dose tested (10^{-6}–10^{-8} M) did fMLP provoke significant LTB_4 secretion (Fig. 3) although the expected increases in cytosolic Ca^{2+} (ED_{50} 10^{-9} M-fMLP) and $O_2^{-\cdot}$ generation (ED_{50} 5×10^{-8} M-fMLP) resulted.

Effect of adding linoleic acid

In order to determine whether neutrophils could form LTB_4 in the presence of another *cis*-unsaturated fatty acid or whether LTB_4 generation was specific in response to arachidonate, were preincubated neutrophils with 50 μM-linoleic acid and then exposed them to STZ. Linoleic acid is not a substrate for 5-lipoxygenase and therefore not a competitive inhibitor of arachidonate metabolism [29]. The concentration of linoleic acid used was capable of activating neutrophils to generate $O_2^{-\cdot}$ to the same degree as 50 μM-arachidonate (Fig. 4). However, linoleic acid did not cause neutrophils to form significant amounts of LTB_4 in the presence of STZ (Table 2).

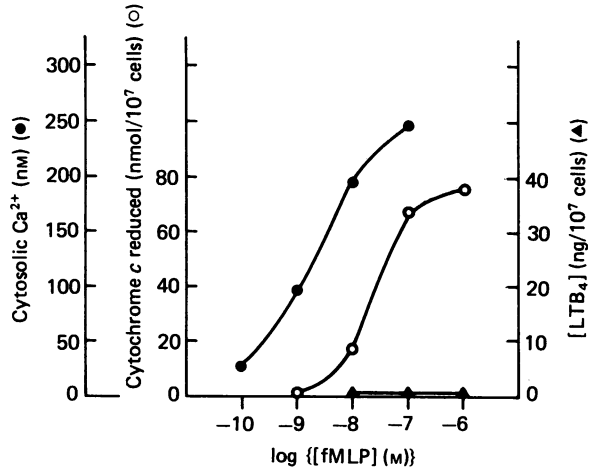

Fig. 3. Dose–response of cytosolic Ca^{2+}, $O_2^{-\cdot}$ generation and LTB_4 secretion by neutrophils treated with fMLP

Changes in cytosolic Ca^{2+} concentration and $O_2^{-\cdot}$ generation were measured by continuous methods (see the Materials and methods section). LTB_4 was measured after 10 min treatment with fMLP (see the Materials and methods section). ●, Cytosolic Ca^{2+}; ○, $O_2^{-\cdot}$ generation; ▲, LTB_4 secretion.

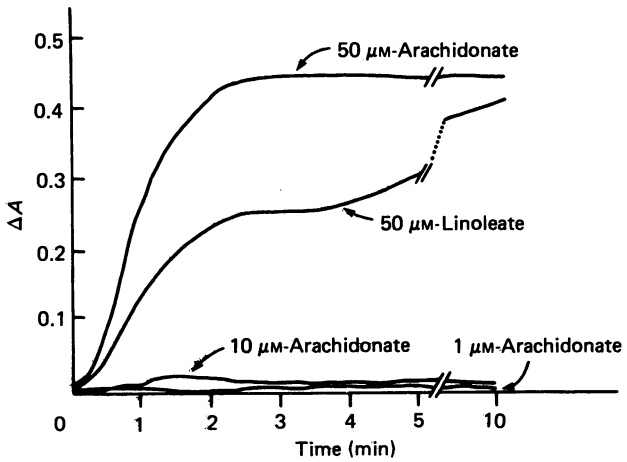

Fig. 4. $O_2^{-\cdot}$ generation by neutrophils stimulated with 50 μM-arachidonic acid or 50 μM-linoleic acid

Neutrophils (4×10^6) were preincubated at 37 °C for 5 min before the addition of fatty acid in ethanol. Ethanol concentration was 0.1%. $O_2^{-\cdot}$ generation was monitored continuously as superoxide dismutase-inhibitable reduction of cytochrome *c* (see the Materials and methods section).

Effect of plasma on STZ-generated LTB_4

It has been reported that the LTA_4 hydrolase is found in plasma [30] and it is therefore possible that, *in vivo*, neutrophils release LTA_4 which can only be converted to LTB_4 in the presence of plasma. While the lack of 5-HETE and all-*trans*-LTB_4 in our extracts made this possibility unlikely, it was formally tested by treating neutrophils with STZ in the presence of 20% plasma. Plasma did not significantly enhance formation of LTB_4 over amounts formed in Hepes buffer (1.2 ng/10^7 cells versus 1.3 ng/10^7 cells, $n = 2$).

DISCUSSION

LTB_4, which was originally isolated from human neutrophils stimulated with the calcium ionophore, A23187 [14,15], is among the most potent mediators of inflammation so far identified. LTB_4 causes neutrophil chemotaxis and degranulation at nanomolar concentrations and $O_2^{-\cdot}$ generation at micromolar concentrations [1–5]. It causes rapid increases in cytosolic calcium concentrations [31], release of membrane-associated calcium [32], and turnover of membrane phospholipids [33], most likely through a receptor-mediated process [34,35]. Because of its potent activity, and because neutrophils are capable of synthesizing prodigious amounts of LTB_4, it has been hypothesized that LTB_4 is an integral part of the acute inflammatory response either as an intra- or extra-cellular mediator [1,7–10].

We therefore attempted to determine which ligand–receptor interactions likely to play a role in inflammation would launch formation of LTB_4 by neutrophils. Cells were exposed to STZ, heat-aggregated IgG, and fLMP, which activate neutrophils via the C3b, Fc, and fMLP receptors, respectively. None of these three ligands, be it soluble, such as fMLP, ingestible particles, such as STZ, or particles too large to phagocytose, such as aggregated IgG, provoked neutrophils to generate significant quantities of the leukotriene. Indeed, the quantities generated in the absence of 50 μM-arachidonic acid are too small to be verified as LTB_4 rather than the double-lipoxygenation compound, 5*S*,12*s*-diHETE, which co-elutes with LTB_4 on reverse-phase h.p.l.c. [17]. While neutrophils stimulated with ionophore make significant amounts of LTB_4 in the absence of added arachidonate, it is possible that LTB_4 is produced in acute inflammation only when arachidonate is released from other cells. Marcus *et al.* [27] have demonstrated that neutrophils can utilize arachidonic acid derived from platelets as a substrate for LTB_4 production. We therefore added incremental amounts of arachidonic acid to neutrophil suspensions and found that arachidonate concentrations far in excess of physiological substrate amounts were required before LTB_4 synthesis commenced. Clancy *et al.* [36] have reached a similar conclusion, demonstrating that neutrophils stimulated with fMLP or C5a synthesized LTB_4 only in the presence of exogenous arachidonic acid (250 μM). They suggest that membrane phospholipases fail to cleave arachidonate under these conditions. However, Walsh *et al.* [37], using STZ, and Wynkoop *et al.* [38], using fMLP, have clearly demonstrated that neutrophils rapidly mobilize membrane arachidonate when stimulated with inflammatory ligands. Therefore, lack of phospholipase activation cannot be responsible for the failure of neutrophils to synthesize LTB_4, which must be due to a separate requirement for activation of the 5-lipoxygenase.

On a stoichiometric basis, both 1 μM- and 10 μM-arachidonic acid are quantities of substrate far in excess of that needed to synthesize the nanomolar concentrations of LTB_4 which are sufficient for cell activation. Moreover, as shown by our experiments with [^{3}H]arachidonate, 70% of the added lipid partitioned into the cells. However, in the presence of up to 10 μM-arachidonic acid, neutrophils treated with STZ did not synthesize significant amounts of LTB_4 or any lipoxygenase product (including 15-HETE; results not shown). In contrast, 50 μM-arachidonic acid added to neutrophil suspensions and subsequent stimulation with STZ resulted in at least 40-fold greater LTB_4 synthesis, as combined ω-metabolites and LTB_4, than in those stimulated with STZ alone. The data suggest that exogenous arachidonic acid does not simply provide a substrate but rather is involved in the activation of the 5-lipoxygenase.

Tripp *et al.* [39] have reported that PMA, which activates PKC, acts in synergy with calcium ionophore to stimulate LTC_4 production by mouse peritoneal macrophages. Liles *et al.* [26] have obtained similar results for LTB_4 with human neutrophils. McPhail *et al.* [12] have reported that *cis*-unsaturated fatty acids, including arachidonic acid, also activate PKC. Since it was likely that arachidonate was acting to stimulate LTB_4 synthesis by some means other than providing substrate, we examined the possibility that its ability to activate PKC might be involved in LTB_4 generation. We therefore examined the effect of PMA and linoleic acid, two other activators of PKC, on STZ-stimulated LTB_4 production by neutrophils. Neither compound, however, activated the lipoxygenase pathway as measured by LTB_4 production.

Dahinden *et al.* [40] have reported that murine mast cells synthesize and secrete LTA_4, which is converted to LTC_4 in plasma, while Fitzpatrick *et al.* [30] have documented the action of a plasma LTA_4 convertase. Were this enzyme required for leukotriene synthesis by neutrophils treated with inflammatory ligands, we would have expected to recover significant quantities of 5-HETE and all-*trans*-LTB_4 products from our neutrophil suspensions. Rather, little or no other products of the 5-lipoxygenase pathway were found, suggesting that this was unlikely. However, we formally tested whether neutrophils exposed to inflammatory ligands would synthesize LTB_4 in the presence of plasma. We treated neutrophils with STZ in the presence of 20% plasma and found no significant increase in LTB_4 over that found when cells were treated with STZ in Hepes buffer.

Many investigators have reported LTB_4 synthesis by neutrophils exposed to fMLP, STZ, C5a, and monosodium urate. There are several reasons why those results may differ from ours. Jubitz *et al.* [41], Claesson *et al.* [42] and Serhan *et al.* [43] required 10^9 neutrophils, or more, to identify any LTB_4 in their preparations. Moreover, it is difficult determine whether monocytes, which do synthesize LTB_4, contaminated these preparations. Palmer & Salmon [44] and Williams *et al.* [45] have relied on radioimmunoassays in which cross-reactivity with 12-HETE or 5*S*-diHETE may confuse the picture in the absence of vigorous exclusion of platelet contamination. The persistence of immunoreactive LTB_4, as seen by Williams *et al.* [45] in cell suspensions after 30 min of

incubation, makes this fact likely as Shak & Goldstein [19] have clearly documented that LTB_4 is fully metabolized to its ω-oxidation products after this time period. 5*S*,12*S*-diHETE, on the other hand, is oxidized much less efficiently.

In summary, our data show that neutrophils do not require exogenous arachidonate to generate large quantities of LTB_4 when stimulated with calcium ionophore. However, neutrophils respond to inflammatory ligands (IgG, C3b, fMLP) by generating significant quantities of LTB_4 only in the presence of non-physiological concentrations of arachidonic acid. This effect of the arachidonate is not shared by linoleic acid (another *cis*-unsaturated fatty acid which activates PKC and provokes generation of $O_2^{-\cdot}$. The mechanism by which high concentrations of arachidonate act in synergy with ligand–receptor interactions to stimulate LTB_4 production remains to be determined. Rouzer & Samuelsson [46] have demonstrated that the 5-HETE enzyme requires multiple cofactors for activation. It is possible that neutrophils are poorly capaple of generating at least one of these cofactors which, *in vivo*, is contributed by another type of cell. This putative cofactor may, for example, mediate arachidonylation of neutrophil proteins. Alternatively, the cofactor may itself be a metabolite of arachidonic acid required to activate either the 5-lipoxygenase or a phorbol-insensitive protein kinase. One obvious candiate for such a role would be lipoxin A, an activator of PKC [47].

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