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Immunology

Leukotriene C_4 production by murine mast cells: Evidence of a role for extracellular leukotriene A_4

(leukotrienes/arachidonic acid/lipoxygenase/neutrophils/cellular interactions)

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ABSTRACT **The glutathione-containing leukotriene C_4 (LTC_4) is a major mediator of smooth muscle contraction and is released by mast cells when antigen interacts with cell-bound IgE. Antigen-stimulated mast cells undergo phospholipase activation. We report a pathway of LTC_4 production by mast cells that does not require phospholipase activation but depends on the interaction of activated neutrophils with unstimulated mast cells, using as an intermediate extracellular leukotriene A_4 (LTA_4). The epoxide LTA_4 is released by neutrophils and, together with leukotriene B_4 and 5-hydroxyicosatetraenoic acid, constitutes the major lipoxygenase metabolites found in supernatants of stimulated neutrophils. Five minutes after activation of neutrophils by calcium ionophore A23187 we measured 136 pmol of extracellular LTA_4 per 10^7 neutrophils (range 40–300, $n = 7$) by trapping the expoxide with alcohols. Therefore, we conclude that LTA_4 is not just an intracellular leukotriene precursor but is released as a lipoxygenase metabolite. LTA_4 is known to be stabilized by albumin and is efficiently converted by mast cells into LTC_4 even at low LTA_4 concentrations. The LTA_4 complexed to albumin is converted into LTC_4 rapidly and completely within 10–15 min. More than 50% of the LTA_4 presented to mast cells is metabolized to LTC_4 at concentrations of LTA_4 between 0.2 and 2 nmol of LTA_4 per 10^7 mast cells. This observation establishes a potential physiologic role for extracellular LTA_4. Therefore, interactions between various cell types that release or utilize LTA_4 may provide an important metabolic pathway for the production of leukotrienes.**

The chemical structure of slow-reacting substance of anaphylaxis, SRS-A, has been established recently by the work of Sammuelsson and Corey, who demonstrated SRS-A to be a family of sulfidoleukotrienes, LTC_4, LTD_4, and LTE_4 (1, 2). Sulfidoleukotrienes are potent mediators of smooth muscle contraction and have been implicated in the pathogenesis of asthma (3). Leukotrienes are produced by mast cells (MCs) after binding of antigen to membrane-bound IgE (3, 4). The metabolic pathway of LTC_4 production proceeds by release of arachidonate from phospholipids, oxidation by 5-lipoxygenase, further oxidation to form LTA_4, and enzymatic conversion of LTA_4 to LTC_4 by a glutathionyltransferase (1, 2). Stimulated polymorphonuclear neutrophils (PMNs) have a very active 5-lipoxygenase pathway (5, 6), but in PMNs the LTA_4 is stereospecifically hydrolyzed to LTB_4, a potent chemotactic factor (5–8). Thus, depending on the cell type and the enzymes they possess, LTA_4 is converted to either SRS-A or LTB_4. The leukotriene precursor LTA_4 is an extremely unstable epoxide, which is spontaneously hydrolyzed to biologically inactive (8) 5,12-dihydroxyicosatetraenoic acid (DiHETE) stereoisomers in aqueous solution (9) under neutral or acidic pH conditions (Fig. 1).

Largely because of its chemical instability, LTA_4 has been considered primarily as an intracellular leukotriene precursor. Its presence was demonstrated in neutrophils after cell activation, but no attempt was made to determine if LTA_4 is released into the extracellular medium (9). Not all of the LTA_4 formed is converted to LTB_4 by leukocytes, since in all cases when LTB_4 is detected in cell extracts one also observes the biologically inactive 5,12-DiHETE stereoisomers (1–3), which are derived from hydrolyzed LTA_4. However, it was not known whether the excess LTA_4 decays intracellularly or if it can be released into the medium.

In this study we examined LTA_4 release from stimulated neutrophils. This question became particularly important since we recently observed that mixed leukocytes produce more LTC_4 than individual purified cells present in the mixture (10). Furthermore, Fitzpatrick *et al.* demonstrated that synthetic LTA_4 is stabilized by albumin (11), a fact indicating that if LTA_4 is released into the medium it could have biologic effects at some distance from the cells producing LTA_4. Using an alcohol trapping procedure, we performed experiments (Fig. 2) showing that LTA_4 is indeed a major lipoxygenase metabolite released by neutrophils. In addition, we show that LTA_4 complexed to albumin is efficiently converted by mast cells to LTC_4 at LTA_4 concentrations commonly found in supernatants of stimulated neutrophils. This establishes a pathway for LTC_4 production through an intermediate of extracellular LTA_4. LTC_4 production resulting from interactions of activated PMNs with MCs promises to be an important pathway of LTC_4 formation independent of phospholipase activation in the mast cell. These observations provide an interpretation for synergistic LTC_4 production by nonhomogeneous cell cultures and may enhance our understanding of the pathogenesis of asthma and the role that LTC_4 plays in inflammation.

METHODS

Cell Preparations and Reaction Conditions. PMNs were isolated from heparinized (preservative-free sodium heparin, 20 units/ml) blood from healthy adult volunteers as described (6), using two purification steps. The isolated leukocytes were >99% PMNs. After two washes in Gey's solution (GIBCO) the cells were suspended in incubation medium consisting of Dulbecco's phosphate-buffered saline supplemented with fatty acid-free bovine serum albumin (Pentex) at 10 mg/ml. The mast cells were obtained by culturing bone

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Abbreviations: SRS-A, slow-reacting substance of anaphylaxis; PMN, polymorphonuclear neutrophil; MC, mast cell; LT, leukotriene; DiHETE, dihydroxyicosatetraenoic acid.

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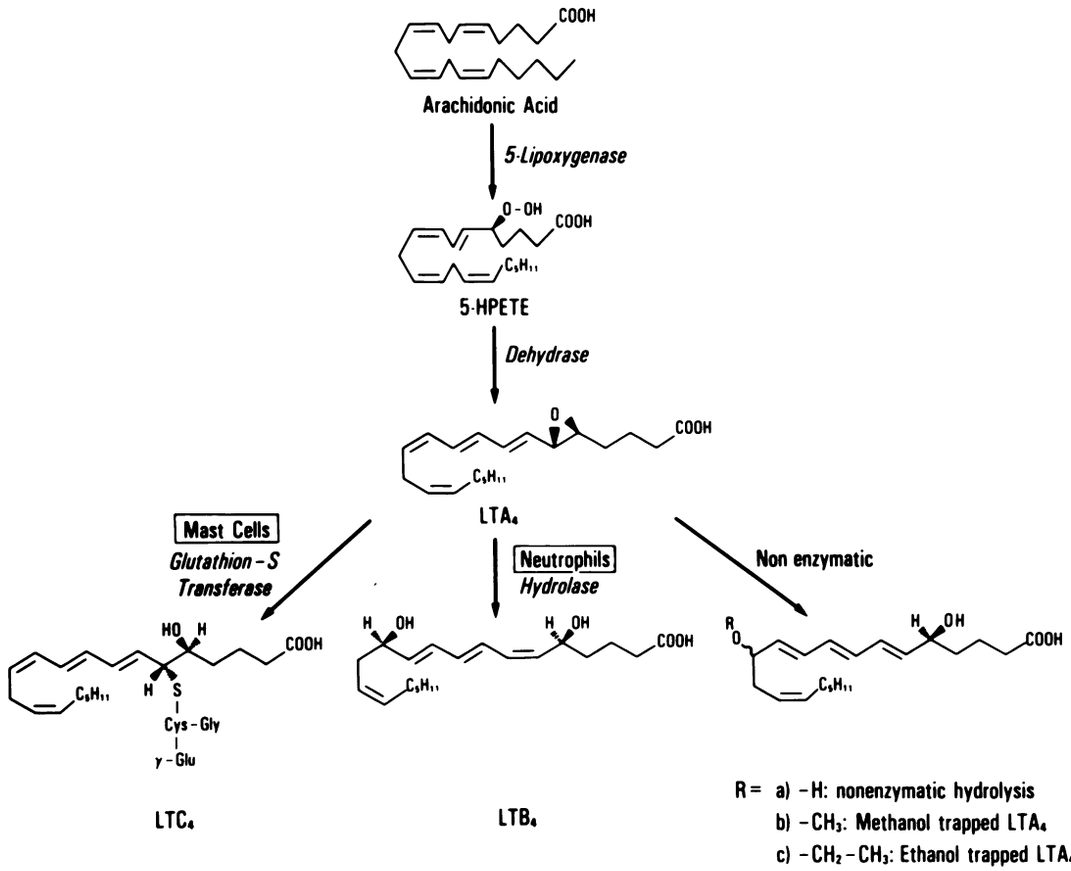

FIG. 1. Arachidonic acid metabolism in PMNs and MCs by the lipoxygenase pathway. The products formed from nonenzymatic hydrolysis of LTA_4 and trapping of LTA_4 by alcohol are also shown (adapted from refs. 1, 2, and 9). 5-HPETE, 5-hydroperoxyicosatetraenoic acid.

marrow cells of BDF_1 mice in Dulbecco's modified Eagle's medium supplemented with heat-inactivated horse serum (10%, vol/vol), and the supernatant from a mouse cell line (WEHI 3) producing interleukin 3 (10%, vol/vol) was added as described (4). Nonadherent cells were selected and subcultured every 3–4 days for 8 weeks. The cell lines recovered were judged to be >95% MCs by staining with toluidine blue. In addition, cells from such lines were positive for IgE receptors and produced LTC_4 after challenge with either IgE–antigen complex (anti-dinitrophenyl IgE plus dinitrophenyl-carrier) or Ca^{2+} ionophore. Mast cells were washed free of media and suspended in phosphate-buffered saline with bovine serum albumin at 10 mg/ml to a final concentration of 10^7 cells per ml. To determine LTC_4 production by MCs from endogeneous arachidonic acid, cells were labeled with tritiated arachidonic acid (86 Ci/mmol, Amersham; 1 Ci = 37 GBq) by incubating 2.5×10^7 MCs overnight in 40 ml of culture medium containing arachidonic acid at 0.5 μCi/ml. The cells were cultured for an additional 3 hr in label-free medium and washed twice before they were used for LTC_4 release experiments.

Lipoxygenase metabolites generated by PMNs were determined after the cells (10^7–10^8 per ml) were incubated at 37°C and challenged with 10 μM Ca^{2+} ionophore A23187 (Calbiochem). At the times indicated, cells were sedimented in a Microfuge for 30 sec and supernatants were harvested. LTA_4 trapping experiments and procedures used in purification of lipoxygenase metabolites generated by the cells were performed as described (9).

Lipid Extraction and Analysis. One milliliter of supernatant (containing 1 nmol of PGB_2 as an internal standard) was mixed with 10 ml of either methanol or ethanol. The alcohol mixture was acidified to pH 3 with 1 M HCl and 25 ml of diethyl ether (Mallinckrodt) was added. Two phases were formed by the addition of 30 ml of water. The organic phase was evaporated to dryness under N_2 and the residue was subjected to silicic acid chromatography as described (5, 6) and the products recovered were analyzed by HPLC.

LTC_4 is produced by MCs exposed to albumin-bound LTA_4. The reaction was initiated by adding 1 vol of LTA_4 complexed to albumin (1% in phosphate-buffered saline) to MCs and the mixture was incubated at 37°C. The reaction was stopped by adding 0.6 vol of isopropropyl alcohol. Sulfidoleukotrienes were extracted as described by Clancy and Hugli (12) and were analyzed by reverse-phase HPLC. Each experiment included two extractions of synthetic LTC_4 as a control and the extraction recovery was as reported (12). The LTA_4 was stabilized in 1% albumin solution (11) and the concentration of LTA_4 recovered from the MCs was verified by trapping experiments with methanol or ethanol using the same procedures as described for the PMN supernatants.

The lipoxygenase metabolites were identified by (*i*) having elution profiles on reverse-phase and straight-phase HPLC identical to those of biosynthetic and synthetic standards in either the free acid or the methyl ester form (8); (*ii*) bioassay (8), (*iii*) UV spectroscopic analysis (5, 8), and (*iv*) gas chromatography/mass spectroscopy analysis (8, 9) of the trimethylsilylated methyl ester derivatives performed as described (13) using a Hewlett–Packard 5981-A GC mass spectrometer.

Four nanomoles of LTC_4 purified by HPLC was hydrolyzed with 6 M HCl at 110°C for 24 hr and amino acid analysis was performed with a Beckman model 121M analyzer. Bioactivity of LTC_4 was assessed as previously described (12).

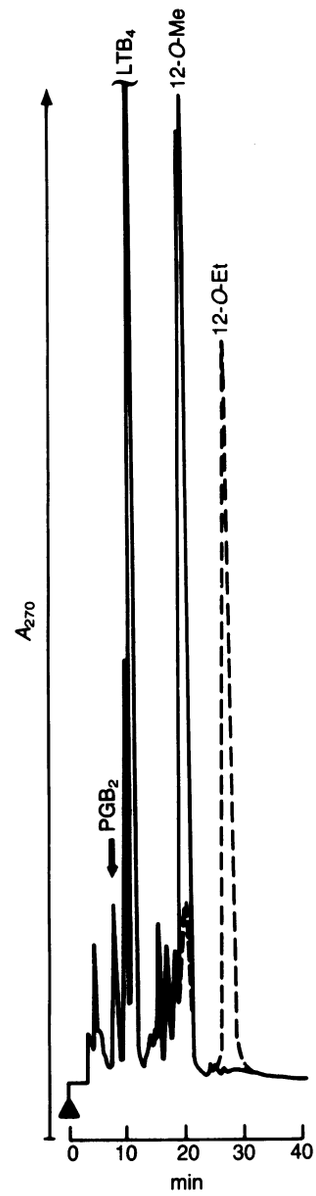

FIG. 2. Demonstration of extracellular LTA_4 in the supernatants of ionophore-activated PMNs. HPLC analysis of the trienes found in 1 ml of supernatant from 10^8 PMNs challenged for 5 min with 10 μM Ca^{2+} ionophore A732187 and trapped with either methanol or ethanol. One nanomole of prostaglandin B_2 (PGB_2) was added as an internal standard. The material eluted from silicic acid by ethyl acetate (5, 6) was separated on a Nucleosil C_{18} column (250 × 4.6 mm; Alltech, Deerfield, IL). The C_{18} column was eluted with methanol/water/acetic acid (75:25:0.01, vol/vol) at a flow rate of 1 ml/min. Absorbance was monitored at 270 nm. The solid line represents the HPLC profile of supernatants trapped with methanol and the interrupted line illustrates an ethanol-trapped sample. The two chromatograms were identical except for the elution position of 12-*O*-methyl and 12-*O*-ethyl derivatives of LTA_4. Retention times (in min) of synthetic trienes shown in the chromatograms were as follows: ω-oxidized leukotrienes, 4.55; PGB_2 (internal standard), 7.81; 6-*trans*-LTB_4, 9.5; 12-epi-6-*trans*-LTB_4, 10.0; LTB_4, 10.8; LTB_4 lactone, 15.0; 5,6-DiHETEs, 16.6 and 18.0; 12-*O*-methyl-6-*trans*-LTB_4 isomers, 19.2 and 19.7; and 12-*O*-ethyl-6-*trans*-LTB_4 isomers, 27.2.

Radioactivity in HPLC fractions containing LTC_4 was measured in 10 ml of Insta-Gel scintillation fluid (United Tech Packard) and β emission of tritium was measured in a Beckman model L58100 scintillation counter.

All lipoxygenase metabolites were stored in methanol under argon at −70°C. Synthetic LTB_4, 20-hydroxy-LTB_4, LTA_4, LTC_4, LTD_4, and LTE_4 were generous gifts of J. Rokach (Merck Frosst Labs, Pointe Claire, PQ).

RESULTS

We recently observed synergistic production of LTC_4 in mixtures of PMNs and MCs (10). In subsequent experiments we found that supernatants from stimulated PMNs induced LTC_4 production by MCs when albumin was present in the medium (data not shown). Since it has been reported that the epoxide LTA_4 is stabilized by albumin (11), we hypothesized that LTA_4 released from the PMNs might be involved in this synergy. To determine the biochemical nature of this cellular interaction, we measured LTA_4 in the supernatant fluids of PMNs challenged with Ca^{2+} ionophore, using methanol and ethanol trapping protocols as previously described (9). As shown in Fig. 2, the epoxide LTA_4 is indeed released from stimulated PMNs with only minimal intracellular nonenzymatic hydrolysis. In preliminary time course experiments, the release of LTA_4 was rapid, preceding LTB_4 release, and was maximal at 2–4 min. A slow decline in extracellular LTA_4 concentration occurs over the next 20 min, suggesting degradation or uptake of lipid. In an extensive study (seven experiments), which employed four separate donors, we detected 40–300 pmol of LTA_4 (mean 136 pmol) in supernatants of 10^7 PMNs after 5 min of incubation at 37°C. LTA_4 was measured as either the 12-*O*-methyl or the 12-*O*-ethyl derivative after alcohol trapping (quantities of LTA_4 trapped by ethanol or methanol vary less than 20% when determined in supernatants from the same cell batch). Borgeat and Samuelsson (14) found that ionophore-stimulated PMNs produced about 0.56 nmol of LTB_4 per 10^7 cells per 4 min. From these data we calculate that as much as 20–30% of the LTA_4 is released from intact cells. Thus, LTA_4 is an important anabolic extracellular product, on the basis of bulk production. It should be noted that these values are minimal estimates, since absolute quantitation of a labile epoxide may not be possible with these methods. No LTA_4 could be trapped in supernatants of neutrophils when albumin was omitted from the medium.

In Fig. 2 we show a chromatogram of a mixture of purified methyl ester derivatives of LTA_4 commonly obtained from neutrophil supernatants. The 5,12-DiHETE stereoisomers were purified from neutrophils incubated in medium devoid of albumin (8). All compounds showed identical UV absorption spectra with λ_{max} at 268 nm and shoulders at 258.5 nm and 279.5 nm indicative of the all-*trans* triene configuration (8). The GC/mass spectra of trimethylsilylated compounds (8) differed from one another as expected. The mass spectrum of each compound corresponded to reported values (5, 8, 9). Common fragment ions were found for all trienes at m/z 171, 189, 203, 293, and 404. Ions with m/z 129, 191, 217, 293, and 383 of LTB_4 shifted by 58 units (trimethylsilyl vs. methyl) to m/z 71, 133, 159, 235, and 325 for the methanol-trapped compound and by 44 units (trimethylsilyl vs. ethyl) to m/z 85, 147, 173, 249, and 339 for the ethanol-trapped compound, as predicted (9). Physicochemical properties of alcohol-trapped LTA_4 isolated from PMN supernatants and the corresponding derivatives of synthetic LTA_4 were identical. From these experiments we conclude that LTA_4 is indeed a major lipoxygenase metabolite released from stimulated PMNs and suggest that a biologic role exists for extracellular LTA_4.

In Fig. 3 we show that synthetic LTA_4, bound to and stabilized by albumin, is efficiently converted to LTC_4 by MCs. The conversion of LTA_4 to LTC_4 is rapid and complete within 10–15 min (Fig. 3*A*). More than 50% of LTA_4 is converted to LTC_4 by 10^7 MCs at LTA_4 concentrations up to 2 nmol/ml (Fig. 3*B*). Most important, the uptake and metabolism of LTA_4 occur at physiologically relevant concentrations. For example, when 100 pmol of LTA_4 in 500 μl of 1% albumin are fed to MCs, 80–87 pmol of LTC_4 is measured

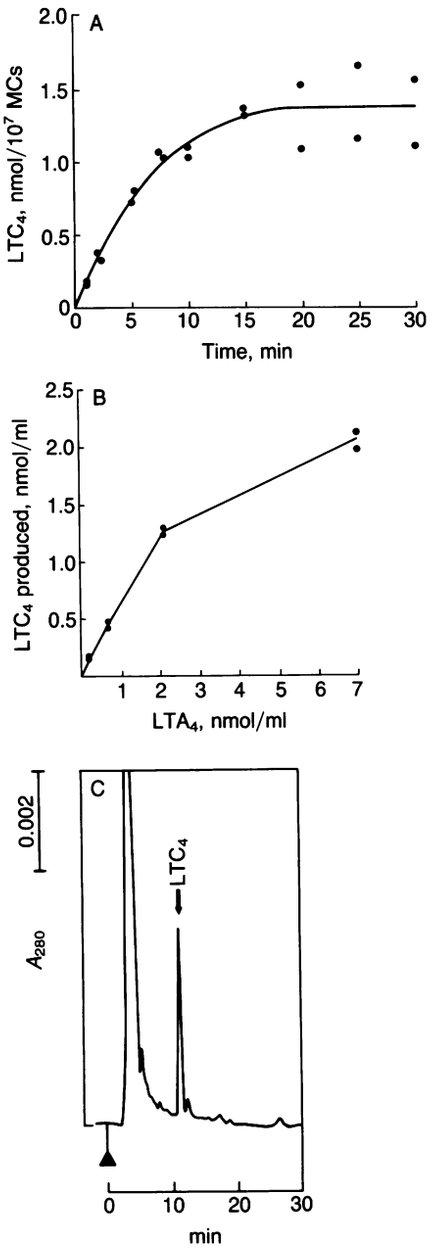

FIG. 3. LTC_4 production by MCs exposed to albumin-bound LTA_4. (*A*) Time course for LTC_4 production by 10^7 MCs (exposed to LTA_4 at 2 nmol/ml) in 1 ml of buffer at 37°C. Conversion of LTC_4 to LTD_4 or LTE_4 was not observed and no LTC_4 was degraded even after 30 min. (*B*) LTC_4 produced by MCs is shown as a function of LTA_4 added. The LTC_4 measurements were taken after 10 min at 37°C. (*C*) HPLC of an extract taken from 5×10^6 MCs incubated for 10 min at 37°C with 100 pmol of LTA_4 complexed to albumin at 10 mg/ml. LTC_4 was separated on a Nucleosil C_{18} column (250 × 4.6 mm; Alltech) eluted with methanol/water (65:35, vol/vol) containing 3.5 mM ammonium acetate at pH 5.7 (apparent pH) and developed at a flow rate of 1 ml/min.

after only 10 min (Fig. 3*C*). No LTD_4 or LTE_4 was detected even after incubation of LTA_4 with MCs for up to 30 min. It is interesting that the HPLC elution profile of products obtained from MCs exposed to albumin-bound LTA_4 was identical to that observed when LTC_4 was formed by MCs using endogeneous arachidonic acid sources and stimulated by Ca^{2+} ionophore or IgE and antigen (data not shown). Small quantities of 11-*trans*-LTC_4 (eluting just after the LTC_4 peak in Fig. 3*C*) were detected in our supernatants, an observation that is in contrast to the large amount of 11-*trans*-LTC_4 formed when the MCs are incubated with high concentrations of LTA_4 dissolved in organic solvents (15).

Material obtained from the MCs incubated with albumin–LTA_4 was purified by extraction and HPLC. The LTC_4 was identified by the following criteria. The cell-derived product had the same retention time on reverse-phase HPLC as synthetic LTC_4 (Fig. 3*C*). The UV spectrum of the MC product was identical to that of synthetic LTC_4 with a λ_{max} of 280 nm, which is distinct from that of 11-*trans*-LTC_4 (λ_{max} = 278 nm; ref. 4) (data not shown). The threshold activity in inducing contraction of the smooth muscle of guinea pig ileum was identical to that of synthetic LTC_4. Contraction was abrogated by the leukotriene antagonist FPL55712 (data not shown). Finally, equimolar quantities of glycine and glutamic acid were detected by amino acid analysis in hydrolysates of cell-derived LTC_4, and no other amino acids were detected.

When radioactive LTA_4 was incubated with MCs it was quantitatively converted to labeled LTC_4 and a linear correlation was found between radioactivity in the HPLC fraction containing LTC_4 and LTC_4 measured by UV absorbance in dose–response studies (data not shown). To demonstrate that LTC_4 formed by mast cells incubated with albumin-bound LTA_4 was derived exclusively from extracellular LTA_4 and not formed from endogenous arachidonic acid, we labeled MCs with tritiated arachidonate. These labeled cells were activated with calcium ionophore and the LTC_4 formed was purified by HPLC and found to be radioactive (95,000 cpm/nmol). When the same labeled cell batch was incubated with albumin–LTA_4, no radioactivity was detected in the HPLC fractions containing LTC_4.

DISCUSSION

In this study we demonstrate a role for extracellular LTA_4 in cellular lipid metabolism. We provide evidence for the release of LTA_4 from stimulated PMNs and we demonstrate efficient metabolism of extracellular LTA_4 into LTC_4 by resting (e.g., unstimulated) MCs. Surprisingly, LTA_4 was found to be a major lipoxygenase metabolite released from activated PMNs. Demonstration that the extremely labile epoxide LTA_4 is released from PMNs was possible only when albumin was included in the medium, since in aqueous buffers LTA_4 is hydrolyzed rapidly to 5,12-DiHETEs (11). Thus, it is reasonable to assume that detection of 5,12-DiHETEs in previous studies may actually reflect LTA_4 release. One might speculate that LTA_4 released from cells such as PMNs could have biological importance other than just being a precursor for LTC_4. Since released LTA_4 is stabilized by albumin, LTA_4 might have biological effects at sites some distance from the neutrophils producing LTA_4. In that light, one could consider LTA_4 along with other arachidonate metabolites as an inflammatory mediator.

We show that mast cells *in vitro* efficiently convert LTA_4 to LTC_4 even when complexed to albumin and at LTA_4 concentrations found in the supernatants of stimulated PMNs. LTC_4 may be produced exclusively from exogeneous LTA_4 without prior activation of phopholipase(s), thus circumventing the use of endogenous arachidonic acid pools. The mechanisms by which MCs convert extracellular LTA_4 into LTC_4 were not investigated in this study. However, the rate and efficiency of the reaction were, and it is intriguing that high-affinity binding of LTA_4 to albumin does not limit cellular uptake. Thus, one may speculate that specific binding sites for LTA_4 exist on MC membranes and compete effectively for the ligand with lipid binding sites on albumin. It should be noted that previous studies have shown that LTA_4 is converted to LTC_4 by MCs and to LTB_4 by both PMNs and erythrocytes (15–17). The earlier studies were designed explicitly to investigate the biochemical pathway of

leukotriene biosynthesis. Large quantities of LTA_4 were dissolved in organic solvents, then added to the cells. Thus, no physiologic implications could be drawn from these studies. It will be important to determine if cells other than MCs can metabolize low levels of LTA_4 bound to albumin and to examine the conversion products from these cells.

Establishing that a particular cell type produces LTC_4 might not be as straightforward as previously assumed. Our data indicate that cell–cell lipid interconversion must be considered when nonhomogeneous cell systems are used. For example, if cell type A is capable of releasing LTA_4 and is contaminated with small or variable numbers of cell type B capable of metabolizing LTA_4 to LTC_4, then the actual quantity of LTC_4 formed will depend solely on the level of LTA_4 produced. If LTC_4 levels correlate with the number of cell type A and not cell type B, and if LTC_4 is released after stimulation with a stimulus specific for cell type A, one may erroneously conclude that cell type A produces LTC_4.

The prominent role of sulfidoleukotrienes in acute allergic reactions has become increasingly clear in recent years (3). Much less is known, however, about their involvement in acute and chronic inflammation. We now know that in the presence of MCs, LTC_4 can be produced by stimulating inflammatory cells such as PMNs; therefore, sulfidoleukotrienes may play a more general role in inflammation than was previously recognized. Since PMNs can destroy sulfidoleukotrienes by producing hypochlorous acid (18), neutrophils may actually play a dual role in modulating formation and control of the LTC_4 levels at an inflammatory site. Thus, activation of the lipoxygenase and LTA_4 release may contribute to LTC_4 production, while activation of the respiratory burst and granule release could limit LTC_4 concentrations at an inflammatory site.

We demonstrated that cells containing enzymes needed for LTC_4 synthesis do not necessarily require activation by a cell-specific stimulus. Thus, SRS-A production induced in lung homogenates by complement component C5a (19) could result from stimulation of LTA_4 release by leukocytes without involving phospholipase activation in mast cells. Furthermore, on the basis of our observations, one may propose a hypothetical scenario for pathogenesis of the elusive late asthmatic response. When IgE–antigen triggers release of LTC_4 and chemotactic factor from MCs, neutrophils are attracted. Neutrophils that arrive in close proximity to MCs may become activated by ingesting MC granules. The activated neutrophils release LTA_4, which in turn is converted to LTC_4 by the MCs, thus provoking a second asthmatic attack. Whatever the *in vivo* function of LTA_4 release and metabolism might be, our studies demonstrate a pathway for LTC_4 production through the interactions of activated neutrophils with resting MCs via extracellular LTA_4.

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