OXIDATIVE DEGRADATION OF LEUKOTRIENE C_4 BY HUMAN MONOCYTES AND MONOCYTE-DERIVED MACROPHAGES

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Human leukocytes can metabolize endogenous arachidonic acid to a variety of biologically active compounds important in the regulation of the immune response. Metabolism via the lipoxygenase pathway results in the synthesis and release of leukotrienes (LT),[1] a unique class of compounds that contain three conjugated double bonds, and which may act as soluble mediators in an inflammatory response (1). LTB_4 has potent effects on the chemotaxis, chemokinesis, and activation of polymorphonuclear leukocytes. LTC_4 and its two derivatives, LTD_4 and LTE_4, comprise the slow-reacting substance of anaphylaxis (SRS-A), which can cause bronchoconstriction and increased vascular permeability. Stimulated neutrophils produce predominantly LTB_4 (2, 3), whereas LTC_4 is a major product of eosinophils (4–6). Human monocytes, when appropriately stimulated, also metabolize arachidonic acid by the lipoxygenase pathway, with the formation of LTB_4 and LTC_4 (7–9).

LT also are metabolized by leukocytes. LTB_4 metabolism by neutrophils is primarily by ω-oxidation (10). Although LTC_4 can be metabolized by sequential peptide bond cleavage to form LTD_4 and LTE_4 (1), LTC_4 also is degraded by the products of the respiratory burst of stimulated phagocytes. Oxidative degradation of LTC_4 by appropriately stimulated eosinophils (11, 12) and normal neutrophils (3, 13, 14) is mediated by the peroxidase–hydrogen peroxide (H_2O_2)–halide system, and the products formed are the chlorosulfonium ion and sulfoxide derivatives of LTC_4, and the *6-trans* isomers of LTB_4 (13, 14). LTC_4 is also degraded by neutrophils that lack peroxidase, i.e., from patients with hereditary myeloperoxidase (MPO) deficiency (3). In this instance, degradation appears to be dependent on the formation of hydroxyl radicals ($OH\cdot$) by the stimulated cells (3, 15).

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[1] *Abbreviations used in this paper:* CGD, chronic granulomatous disease; DMSO, dimethyl sulfoxide; FMLP, *N*-formyl-methionine-leucine-phenylalanine; HETE, hydroxyeicosatetraenoic acid; HPLC, high-performance liquid chromatography; IFN, interferon; LT, leukotriene; MPO, myeloperoxidase; PD, calcium- and magnesium-free phosphate-buffered saline; PMA, phorbol myristate acetate; SRS-A, slow-reacting substance of anaphylaxis.

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Monocytes possess a granule peroxidase identical to the MPO of neutrophils, and respond to stimulation with a respiratory burst leading to the formation of H_2O_2 (16). Monocytes, derived from peripheral blood and maintained as adherent cells in culture, undergo a variety of biochemical and morphologic changes, and differentiate into cells resembling tissue macrophages. During this transformation in culture, there is a progressive loss of the granule peroxidase (17, 19); H_2O_2 release, following a rise at day 3, also falls sharply (20). Treatment of monocyte-derived macrophages with γ-interferon (IFN-γ) results in their activation, with a concommitant increase in H_2O_2 generation when stimulated (21).

These changes in two key components (MPO, H_2O_2) of a system previously demonstrated to degrade LTC_4 might be expected to be reflected in the ability of mononuclear phagocytes to degrade LTC_4. In this study, we report that freshly isolated human monocytes are capable of degrading LTC_4 when stimulated, and that this degradative activity is markedly decreased during time in culture, unless MPO is added. IFN-γ supplementation of the medium during days 6–9 in culture augmented LTC_4 degradation in the presence but not in the absence of added MPO.

Materials and Methods

Special Reagents. Phorbol myristate acetate (PMA) and Histopaque 1077 were obtained from Sigma Chemical Co. (St. Louis, MO); the calcium ionophore, A23187, was obtained from Calbiochem-Behring (San Diego, CA); superoxide dismutase (lyophilized powder from bovine erythrocytes; 12,300 U/mg) from Miles Laboratories, Inc. (Elkhart, IN); zymosan from Nutritional Biochemicals (Cleveland, OH); *N*-formyl-methionine-leucine-phenylalanine (FMLP) from Peninsula Laboratories (Belmont, CA), 14,15-[^{3}H(N)]LTC_4, 35.7 Ci/mmol from New England Nuclear (Boston, MA); and catalase (from bovine liver; 60,000 U/mg) from Worthington Biochemical Corp. (Freehold, NJ). The catalase was dialyzed against sterile nonpyrogenic water before use. RPMI 1640 with Hepes buffer was obtained from Whittaker M. A. Bioproducts (Walkersville, MD), and supplemented with L-glutamine (0.2 mM), penicillin (25 U/ml) and streptomycin (25 μg/ml) (Gibco Laboratories, Grand Island, NY). MPO was purified from canine pyometrial pus by the method of Agner (22) to the end of step 6, and assayed by guaiacol oxidation (23). For the preparation of autologous or AB serum, whole blood was allowed to clot for 30–60 min at room temperature, and the supernatant was collected after centrifugation and frozen at −70°C until use. Synthetic LTC_4 was kindly provided by J. Rokach, Merck Frosst Laboratories (Pointe-Claire/Dorval, Quebec, Canada). 5-hydroxyeicosatetranoic acid (HETE) and 12-HETE were from W. Hubbard, National Institutes of Health (Bethesda, MD), and recombinant human IFN-γ was provided by P. Trown, Hoffman-LaRoche (Nutley, NJ). IFN-γ activity was determined by the supplier in a WISH/VSV cytopathic effect microtiter assay standardized with NIH human IFN-γ reference standard Gg 23-901-530.

All media and buffers were prepared in sterile, nonpyrogenic water (Travenol Laboratories, Inc. (Deerfield, IL), and were filtered before use with 0.22-μm filter units (Sybron/Nalge (Rochester, NY). All glassware was heated at 170°C for 24 h to destroy endotoxin.

Preparation of Monocyte Cultures. Venous blood was obtained after informed consent from 14 healthy human volunteers, a patient with hereditary MPO deficiency, and a patient with chronic granulomatous disease (CGD), all of whom had not taken any medication, including aspirin or other nonsteroidal antiinflammatory agents, for 1 wk. Immediately after collection, the anticoagulated (0.5% K-EDTA) whole blood was diluted 1:1 (vol/vol) with cold calcium and magnesium–free phosphate-buffered saline (PD), and centrifuged at 125 *g* for 20 min at 4°C. The cells were washed twice with PD, resuspended

in warm PD containing 0.3 mM Na-EDTA, underlayered with cold Histopaque 1077 (1.5:1, cell suspension/Histopaque, vol/vol), and centrifuged at 800 *g* at 23°C for 25 min (24). Mononuclear cells removed from the PD/Histopaque interface were washed twice with PD at 23°C and resuspended in RPMI 1640 with 15% autologous serum (or in a few instances, AB serum) at a concentration of 2–3 × 10^6 monocytes/ml. Viability was >92%, as judged by trypan blue exclusion. Monocytes added to sterile 60 × 15 mm tissue culture plates (Costar, Cambridge, MA) in a 3–4-ml volume were allowed to adhere for 2 h in humidified 5% CO_2/95% air at 37°C. To remove nonadherent cells, plates were washed 6–8 times with warm sterile phosphate-buffered saline containing calcium and magnesium (PBS), and fresh RPMI with 15% autologous serum was added. The cultures were maintained in 5% CO_2/95% air with humidity at 37°C, with fresh media added the day after isolation and every other day thereafter. Some monolayers were stained with Diffquik (Difco Laboratories, Detroit, MI) for determination of morphologic detail, and for peroxidase (25). Cell number was determined with an ocular micrometer.

Monolayers supplemented with IFN-γ had fresh medium containing recombinant human IFN-γ at 100 U/ml added on days 6, 7, and 8. Control monolayers had fresh medium alone added at similar times. PMA stimulation was performed on day 9.

Cell Incubations. At specified intervals after adherence, monolayers were washed 6–8 times with warm sterile Tyrode's buffer. Reaction mixtures, containing the components described in the Figures and Tables, were added in a final volume of 3 ml of Tyrode's buffer, and the tissue culture plates were incubated at 37°C in 5% CO_2/95% air for 30 min, unless otherwise specified. All stimuli except zymosan were stored in dimethyl sulfoxide (DMSO) at −70°C, and were diluted with Tyrode's buffer immediately before use. The final concentration of DMSO was 0.02%. At the end of the incubation, the reaction mixtures were removed from the plates and centrifuged at 200 *g* for 10 min at 10°C. The supernatants were extracted on 1 ml octadecyl Baker-10 columns (J. T. Baker Chemical Co., Phillipsburg, NJ), and stored at −20°C until further processing. Samples were evaporated to dryness under nitrogen, suspended in methanol/water/acetic acid (75:25:0.01, vol/vol/vol), pH 4.7, centrifuged at 3,000 *g* for 5 min at 4°C, and the clear supernatant was applied to a 4.6 × 250 mm Ultrasphere ODS C_{18} column (5 μm particle size; Beckman Instruments, Inc., Berkeley, CA). Reverse-phase high-performance liquid chromatography (HPLC) was subsequently performed using the same solvent at a flow rate of 1 ml/min (3). Peaks at 235 nm and 280 nm, which coeluted with authentic LT and HETE standards, were rechromatographed on a 4.6 × 75 mm Beckman Ultrasphere ODS C_{18} column (3 μm particle size), using the same solvent at a flow rate of 1.6 ml/min. Identified peaks were quantitated as previously described (3). Radioactivity was determined with a Beckman model LS 100 C liquid scintillation counter.

Statistical Analyses. Data from different experiments were combined and reported as the mean ± SE. Student's two-tailed *t*-test for independent means was used to analyze differences for significance ($p > 0.05$ considered not significant).

Results

Characteristics of the Monolayers. >98% of the cells of freshly (2 h) adherent monolayers had typical monocyte morphology when examined by light microscopy using Diffquik staining; >95% of the cells were peroxidase-positive. No neutrophils were observed in these preparations.

To assess the degree of platelet contamination (7, 8), supernatants from A23187-stimulated cell preparations were analyzed by HPLC for 5-HETE and 12-HETE, which are products of monocytes and platelets, respectively. When mononuclear suspensions (before adherence) were stimulated with A23187 (1 μg/ml), more 12-HETE than 5-HETE was formed, with the 5-HETE/12-HETE ratio being 0.3 ± 0.5 ($n = 7$). The 12-HETE level was considerably reduced when monolayers were stimulated after adherence for 2 h,

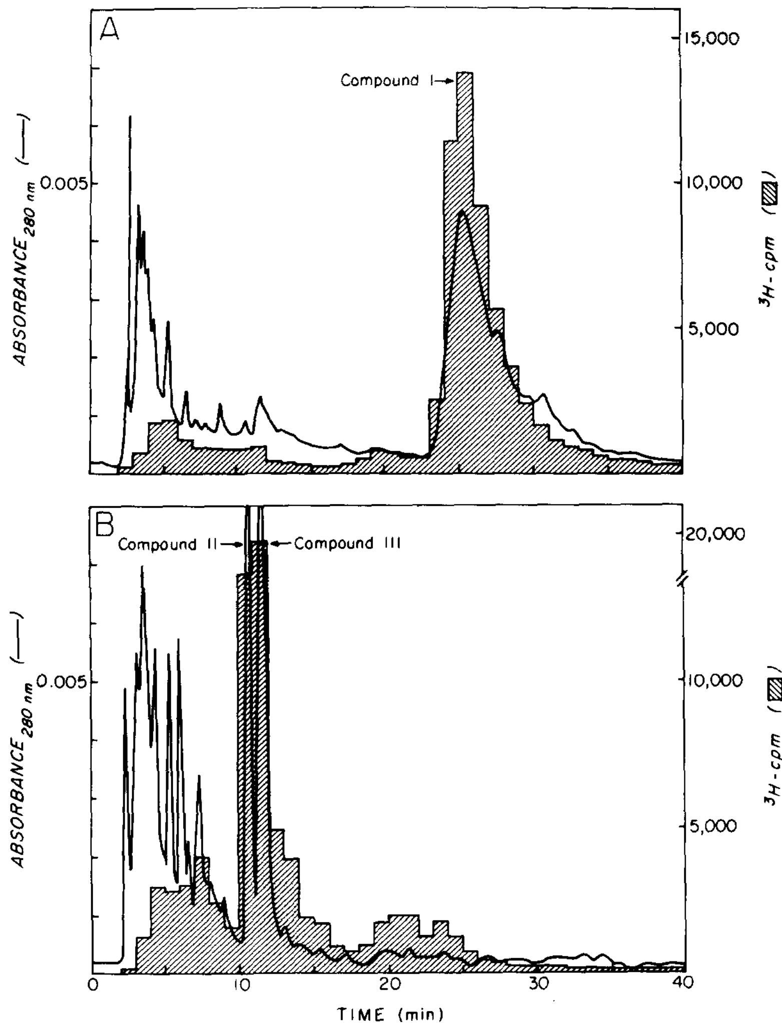

FIGURE 1. LTC_4 degradation by normal monocytes. 2-h adherent human monocytes (5–8 × 10^6) were incubated in 3 ml of Tyrode's buffer containing 400 ng LTC_4 (100,000 cpm of [^3H]LTC_4) in the absence (*A*) or presence (*B*) of PMA (1 μg/ml) for 30 min at 37°C in 5% CO_2/95% air. The reaction mixtures were analyzed by HPLC, as described in Materials and Methods, and sequential 1-ml eluates were assayed for [^3H]LTC_4. Compound I is LTC_4, compound II is 5-(*S*),12-(*R*)-6-trans-LTB_4, and compound III is 5-(*S*),12-(*S*)-6-trans-LTB_4.

with the 5-HETE/12-HETE ratio increasing to 5.5 ± 1.1 (n = 7). The 18-fold increase in the ratio of 5-HETE/12-HETE was taken as evidence of platelet loss during the adherence process.

Degradation of LTC_4. When monocytes, isolated from freshly drawn human blood and allowed to adhere to plastic tissue culture dishes for 2 h, were incubated with LTC_4 for 30 min, little degradation of the added LTC_4 occurred (Fig. 1*A* and Table I). In contrast, when the monolayers were stimulated with PMA (1 μg/ml), 88.9% of the added LTC_4 was degraded (Fig. 1*B* and Table I). The major degradation products were the two all-*trans*-isomers of LTB_4, 5-(*S*),12-

TABLE I

Effect of Inhibitors on LTC_4 Degradation by Normal Human Monocytes

Supplements	LTC_4 degradation (%)*	Significance (p) vs. control	Significance (p) vs. PMA-stimulated cells
None	8.6 ± 1.0 (4)	—	<0.001
PMA (1 μg/ml)	88.9 ± 4.2 (6)	<0.001	—
PMA + catalase (60 μg/ml)	7.0 ± 7.0 (2)	NS[‡]	<0.001
PMA + catalase (heated)	42.5 ± 7.5 (2)	<0.01	<0.002
PMA + azide (10^{-4} M)	0 ± 0 (2)	NS	<0.001
PMA +- SOD[§] (5 μg/ml)	81.0 ± 1.0 (2)	<0.001	NS

2-h adherent monocyte cultures were incubated with LTC_4 as described in Fig. 1, except that the supplements were added at the concentrations shown. Catalase was heated at 100°C for 15 min where indicated.

* Mean ± SE. Number of experiments (n) given in parentheses.

‡ NS, not statistically significant.

§ SOD, superoxide dismutase.

(R)-6-*trans*-LTB_4, and 5-(S),12-(S)-6-*trans*-LTB_4, which together accounted for 46.8% of the radioactivity of the added [^{3}H]LTC_4 (Fig. 1*B*). The degradation of LTC_4 by PMA-stimulated monocytes was greatly inhibited by the hemeprotein inhibitor, azide, and by catalase, which scavenges H_2O_2; heat treatment of the catalase decreased but did not abolish its inhibitory effect. Degradation was unaffected by superoxide dismutase (Table I). These findings suggest that the degradation of LTC_4 by PMA-stimulated monocytes is due to the action of the MPO-H_2O_2 system. Studies with MPO-deficient monocytes supported this conclusion (Fig. 2). 2-h adherent MPO-deficient monocytes did not degrade LTC_4 when stimulated by PMA under conditions in which normal monocytes were highly effective (Fig. 2*A*). The addition of purified MPO to the MPO-deficient monocytes before stimulation by PMA resulted in the complete degradation of the added LTC_4 (Fig. 2*B*). This effect was abolished by heating the MPO before adding it to the reaction mixture. As with normal monocytes, the major degradation products of LTC_4 produced by PMA-stimulated MPO-deficient monocytes supplemented with MPO were the two all-*trans* isomers of LTB_4. Freshly isolated monocytes from a patient with CGD did not degrade added LTC_4 either in the presence or absence of stimulation with PMA (data not shown).

Stimuli of the respiratory burst other than PMA were also capable of inducing LTC_4 degradation of 2-h adherent monocytes (Table II). The calcium ionophore A23187 increased degradation from 8.6% to 27.2%, and the synthetic peptide FMLP, when combined with cytochalasin B, produced 30.9% degradation. Cytochalasin B or FMLP alone at the concentrations used had no effect. Opsonized zymosan centrifuged onto the monolayer produced 71.6% degradation of the added LTC_4, as compared to 16.9% for the centrifuged control. DMSO, which was used as the solvent for all of the stimuli except opsonized zymosan, had no effect on LTC_4 recovery from unstimulated monolayers at the concentration used (0.02%).

Human monocytes maintained in culture gradually lost their ability to degrade LTC_4 (Fig. 3). Degradation by monolayers stimulated with PMA for 30 min decreased from 88.9% at 2 h, to 52.5% at 1 d, to 7.5% at 3 d, and to 0% at 5 d.

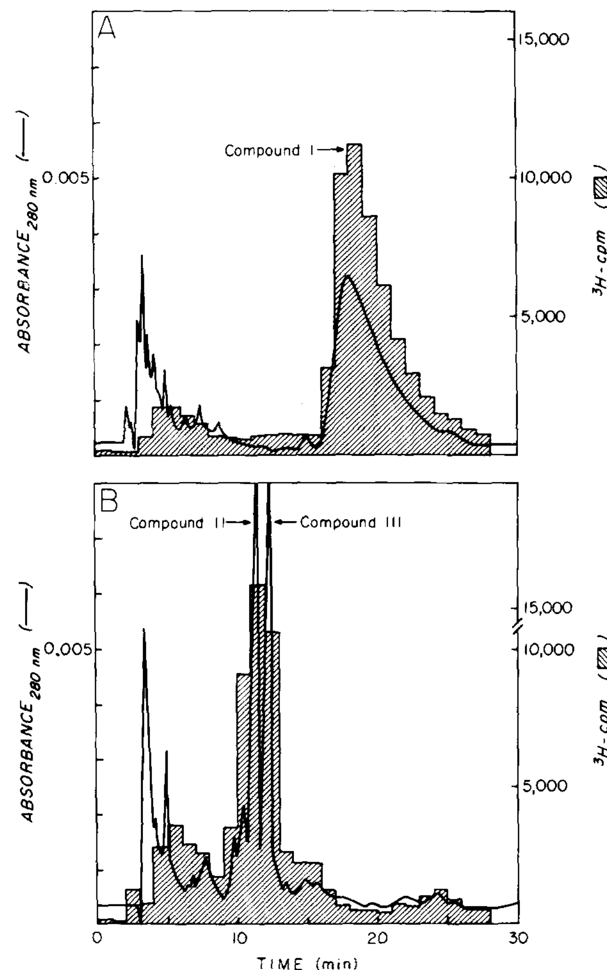

FIGURE 2. LTC_4 degradation by MPO-deficient monocytes. The monolayer cultures in both (*A*) and (*B*) were incubated with PMA as described in Fig. 1, except that MPO-deficient monocytes were used and 228 mU/ml of MPO were added in (*B*).

This decrease in LTC_4 degradation by monocytes with time in culture correlated with a loss in endogenous peroxidase activity (20). When MPO was added to the reaction mixture, stimulation of 3-d adherent monocytes for 30 min with PMA resulted in the complete degradation of added LTC_4 (Fig. 2), with 92% of the radioactivity added as LTC_4 being degraded to the two *all-trans* isomers of LTB_4. The addition of MPO to control cultures (i.e., in the absence of PMA) on day 3 increased LTC_4 degradation from 7.5 to 14.8%. At 5 d in culture, addition of MPO resulted in 55% degradation of LTC_4 under these conditions.

IFN-γ increases H_2O_2 production by stimulated monocyte-derived macrophages (21). Supplementation of the culture medium with recombinant human IFN-γ for 3 d before stimulation with PMA on day 9 had no effect on LTC_4 degradation (Table III). However, when MPO was added to the reaction mixture, IFN-γ supplementation significantly augmented LTC_4 degradation by PMA-

TABLE II
Effect of Various Stimuli on LTC_4 Degradation by Normal Human Monocytes

Stimuli	LTC_4 degradation (%)*	Significance (p) vs. control‡
None (uncentrifuged)	8.6 ± 1.0 (4)	—
PMA (1 μg/ml)	88.9 ± 4.2 (6)	<0.001
A23187 (1 μg/ml)	27.2 ± 4.6 (3)	<0.01
FMLP (10^{-6} M)	7.5 ± 3.2 (2)	NS
FMLP (10^{-5} M)	7.3 ± 1.1 (2)	NS
FMLP (10^{-6} M) + cytochalasin B (10^{-5} M)	30.9 ± 8.2 (3)	<0.05
None (centrifuged)	16.9 ± 1.4 (2)	—
Zymosan (opsonized; 1 mg/ml)	71.6 ± 9.2 (2)	<0.01

2-h adherent monocyte cultures were incubated with LTC_4 as described in Fig. 1, except that the stimuli indicated above were employed. Zymosan, opsonized by incubation with pooled normal human serum for 15 min at 37°C and washed free of unbound serum components, was centrifuged onto the monolayer (125 *g* for 5 min at 23°C) just before incubation; control monolayers were centrifuged under identical conditions in the absence of added zymosan where indicated.

* Mean ± SE. Number of experiments (*n*) given in parentheses.

‡ Opsonized zymosan was compared to the centrifuged control. NS, not statistically significant.

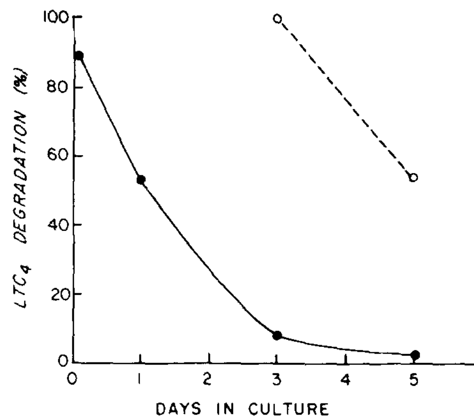

FIGURE 3. Degradation of LTC_4 by normal human monocyte-derived macrophages. Monolayers maintained in culture for the periods indicated were incubated with LTC_4 and PMA as described in Fig. 1 (●). Where indicated (○), 228 mU/ml of MPO were added at the beginning of the 30 min stimulation period.

stimulated macrophages, increasing degradation from 69.5 to 95.6%. The major degradation products were the two all-*trans* isomers of LTB_4.

Discussion

We report here that freshly isolated normal human monocytes maintained as monolayer cultures degrade exogenous LTC_4 when stimulated by PMA, and that degradation occurs by a mechanism involving both MPO and H_2O_2. The evidence for the latter is as follows: (*a*) Degradation was inhibited by azide (which

TABLE III
Effect of IFN-γ on LTC_4 Degradation by Normal Monocyte-derived Macrophages

Supplements	LTC_4 degradation (%)*		Significance (p)‡
	Without IFN	With IFN	
PMA	6.5 ± 6.4 (2)	4.8 ± 0.25 (2)	NS
PMA + MPO	69.5 ± 5.9 (2)	95.6 ± 0.75 (2)	<0.05

Adherent mononuclear phagocytes maintained in culture for 6 d were supplemented with 100 U/ml of recombinant human IFN-γ for 3 d, where indicated. On day 9, the monolayers were stimulated with PMA, as described in Fig. 1, in the presence or absence of MPO (228 mU/ml).
* Mean ± SE. Number of experiments (n) given in parentheses.
‡ Significance between cells cultured in the presence and absence of IFN-γ.

can inhibit MPO) and by catalase (which degrades H_2O_2), but not by heated catalase. (*b*) MPO-deficient monocytes did not degrade added LTC_4 when stimulated unless purified MPO was added. (*c*) CGD monocytes (which lack a respiratory burst and thus do not form H_2O_2) did not degrade LTC_4. (*d*) The cell-free MPO-H_2O_2-halide system rapidly degrades LTC_4 (11, 13, 14).

In contrast to our findings with MPO-deficient monocytes, neutrophils from patients with MPO deficiency degrade LTC_4 when stimulated, and OH· generated by the Haber-Weiss reaction appears to be involved in this degradation (3). This difference may be due to the higher cell numbers (2.0×10^7) used in the neutrophil studies than in the monocyte studies reported here ($5-8 \times 10^6$), and to the considerably greater respiratory burst of neutrophils as compared with monocytes on a per-cell basis (26).

The calcium ionophore, A23187; the chemotactic factor, FMLP, when combined with cytochalasin B; and the phagocytosable particle, opsonized zymosan, could replace PMA as the monocyte stimulus. The major products of LTC_4 degradation by normal human monocytes after incubation with each stimulus were the two all-*trans* isomers of LTB_4, 5-(*S*),12-(*R*)-6-*trans*-LTB_4 and 5-(*S*),12-(*S*)-6-*trans*-LTB_4. These metabolites were also produced when MPO-deficient monocytes supplemented with MPO were used. Other metabolites of LTC_4, namely the chlorosulfonium ion and sulfoxide derivatives, have been proposed (13, 14) as products of the oxidation of LTC_4 by the peroxidase system, and our studies do not exclude their formation by stimulated monocytes. In one study (9) in which the degradation of LTB_4 and LTC_4 by calcium ionophore A23187–stimulated monocytes was assessed, no catabolism was detected. The numbers of monocytes per monolayer in our study was 2–3 times that employed by Williams et al. (9), which may account for their inability to detect LTC_4 degradation by ionophore-stimulated human monocytes.

After 3 d in culture as adherent cells, normal monocyte-derived macrophages lose their ability to degrade LTC_4. The loss of this degradative capacity is in parallel with the loss of granule peroxidase from the cells (20). Although the amount of H_2O_2 produced by stimulated monocyte-derived macrophages is maximal by the third day in culture (20), our studies suggest that the amount produced is insufficient to degrade LTC_4 in the absence of MPO. When stimulated cells are supplemented with MPO on day 3, complete degradation of LTC_4 occurs. At later times (days 5 and 9) under these same conditions, only ~60% of

the added LTC_4 is degraded by stimulated cells, and this may be due to the reduction in H_2O_2 production as these cells mature.

IFN-γ is a potent cytokine, which activates macrophages. One of the consequences of the activation of human monocyte-derived macrophages is an increase in H_2O_2 production by stimulated cells (21). Supplementation of the culture medium of monocyte-derived macrophages with recombinant IFN-γ had no effect on LTC_4 degradation in the absence of peroxidase. However, the addition of MPO to PMA-stimulated cells produced a significantly greater degradation of LTC_4 when the monolayers were pretreated with IFN-γ.

The degradation of LTC_4 by the peroxidase-H_2O_2-halide system yields products with considerably less SRS-A activity than the parent compound (11, 13, 14, 27, 28). Peroxidase-containing phagocytes, i.e., neutrophils, eosinophils, and monocytes, would be expected to directly influence SRS-A activity through the release of their peroxidase and H_2O_2. Modulation of leukotriene activity in an inflammatory lesion might also occur through the reaction of released peroxidase with H_2O_2 generated by a peroxidase-negative phagocyte such as the macrophage. Amplification of this latter effect could occur if the H_2O_2-generating capacity of the macrophage is increased by activation with IFN-γ.

Summary

Freshly isolated 2-h adherent normal human monocytes, when stimulated, degrade added leukotriene C_4 (LTC_4) by a myeloperoxidase (MPO) and H_2O_2–dependent mechanism. Among the stimuli effective in this regard are phorbol myristate acetate (PMA), the calcium ionophore A23187, opsonized zymosan, and *N*-formyl-methionine-leucine-phenylalanine (FMLP) when combined with cytochalasin B. The predominant products formed are the all-*trans* isomers of LTB_4, 5-(*S*),12-(*R*)-6-*trans*-LTB_4 and 5-(*S*),12-(*S*)-6-*trans*-LTB_4. Degradation is inhibited by azide and catalase, but not by superoxide dismutase. LTC_4 degradation does not occur when MPO-deficient monocytes are used, unless MPO is added. Stimulated monocytes from patients with chronic granulomatous disease also are unable to degrade LTC_4 under these conditions. Normal monocytes maintained in culture lose their ability to degrade LTC_4. The addition of MPO to monocyte-derived macrophages increases degradation, particularly when the monolayers are pretreated with γ-interferon. The oxidative degradation of LTC_4 is a capacity shared by neutrophils, eosinophils, and mononuclear phagocytes, and may be an important mechanism for the modulation of leukotriene activity in inflammatory lesions.

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