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Research Article

Polymorphonuclear leukocytes (PMNL) isolated from two patients with chronic granulomatous disease (CGD) were tested for their ability to metabolize arachidonic acid to lipoxygenase products including 5(S),12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid (LTB₄). Analyses of incubations of these PMNL with arachidonic acid and the calcium ionophore A23187 did not differ from simultaneous controls in the production of LTB₄, other 5,12-dihydroxy-eicosatetraenoic acids, or monohydroxy-eicosatetraenoic acids. The clinical diagnosis of CGD was confirmed in both cases by determination of PMNL chemiluminescence. Leukocytes from both patients failed to generate active oxygen species in response to either LTB₄ or formyl-methionyl-leucyl-phenylalanine. The observation of arachidonic acid oxidation in the absence of superoxide anion precludes a role for the active oxygen species in this metabolic process. These studies clearly dissociate the ionophore-induced leukocyte respiratory burst from the oxidation of arachidonate to the leukotrienes. In addition, the defect of CGD appears to be unrelated to the ability of PMNL to carry out arachidonate oxygenation.

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Leukotriene Biosynthesis by Polymorphonuclear Leukocytes from Two Patients with Chronic Granulomatous Disease

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ABSTRACT Polymorphonuclear leukocytes (PMNL) isolated from two patients with chronic granulomatous disease (CGD) were tested for their ability to metabolize arachidonic acid to lipoxygenase products including 5(S),12(R)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid (LTB₄). Analyses of incubations of these PMNL with arachidonic acid and the calcium ionophore A23187 did not differ from simultaneous controls in the production of LTB₄, other 5,12-dihydroxy-eicosatetraenoic acids, or monohydroxy-eicosatetraenoic acids.

The clinical diagnosis of CGD was confirmed in both cases by determination of PMNL chemiluminescence. Leukocytes from both patients failed to generate active oxygen species in response to either LTB₄ or formyl-methionyl-leucyl-phenylalanine. The observation of arachidonic acid oxidation in the absence of superoxide anion precludes a role for the active oxygen species in this metabolic process. These studies clearly dissociate the ionophore-induced leukocyte respiratory burst from the oxidation of arachidonate to the leukotrienes. In addition, the defect of CGD appears to be unrelated to the ability of PMNL to carry out arachidonate oxygenation.

INTRODUCTION

Chronic granulomatous disease (CGD)¹ is characterized by a defect in the polymorphonuclear leukocyte (PMNL) production of superoxide anion (1), hydrogen peroxide, singlet oxygen, and hydroxyl radicals (2, 3). These oxygen species contribute to leukocyte-mediated bacterial killing (2, 3) and their absence is generally believed to account for the impaired ability of patients with CGD to resist certain bacterial infections (4).

In addition to a bactericidal activity, active oxygen species can stimulate the peroxidation of unsaturated lipids. Superoxide-generating systems have been used to oxidize plasma lipids (5) as well as arachidonic acid *in vitro* (6, 7) to produce chemotactic substances. In a careful chemical analysis of arachidonic acid cooxidation by superoxide-dependent oxidants, Fridovich and Porter (7) found 5-hydroperoxy-eicosatetraenoic acid to predominate. 5-Hydroperoxy-eicosatetraenoic acid can also be generated enzymatically by human PMNL (8). This hydroperoxide is further converted via an unstable epoxide to the leukotrienes (9), a novel group of metabolites which appear to play an important

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¹ *Abbreviations used in this paper:* CGD, chronic granulomatous disease; HPLC, high performance liquid chromatography; LTB₄, leukotriene B₄; 5(S),12(R)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; PMNL, polymorphonuclear leukocytes; 5(S),12(S)-DHETE, 5(S),12(S)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid.

role in inflammation. 5(S),12(R)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid (leukotriene B₄; LTB₄) has been shown to stimulate human PMNL chemotaxis (10, 11), degranulation (12), and adherence (13). Other leukotrienes have potent biological activities as well, including leukotriene C₄ and its metabolites, previously known as the slow-reacting substances (see reference 14 for review).

Since leukocytes synthesize oxidized lipids which are chemotactically potent as well as activated oxygen species at levels sufficient to oxidize unsaturated fatty acids, a strong parallel exists between the *in vitro* systems mentioned above and the stimulated leukocyte. These data suggest that a superoxide-dependent oxidant may directly attack arachidonate to yield the hydroperoxy acid precursors to the leukotrienes. This chemical oxidation has been calculated to adequately account for the total production of oxidized lipids by PMNL under certain conditions (15). Alternatively, the production of the hydroperoxy acid leukotriene precursors has, itself, been proposed to be a significant source of active oxygen species through decomposition of the peroxide (16). The absence of superoxide anion production in the leukocytes of CGD patients, therefore, offers a unique opportunity to resolve the interaction between this active molecule (and its consequent oxidants) and arachidonic acid lipoxygenation. In these studies, PMNL were obtained from two patients previously characterized as suffering from CGD. The metabolic activity of these cells in oxidizing arachidonic acid to leukotrienes was assessed. At the same time, the ability of these leukocytes to generate superoxide anion in response to LTB₄ stimulation was determined in a chemiluminescence assay.

METHODS

Patients. Patient 1, a 30-yr-old male, has suffered from recurrent severe bacterial infections (primarily due to *Staphylococcus aureus*) since childhood (17, 18). Previous studies have shown that his PMNL do not reduce nitroblue tetrazolium nor do they increase their oxygen consumption upon stimulation. These cells exhibited reduced bactericidal capacity, but had normal staining characteristics for myeloperoxidase and alkaline phosphatase.

Patient 2, a 37-yr-old male, had recurrent pneumonias and sinusitis during childhood. His major adult infections have been recurrent or chronic gingivitis and skin infections. Previous studies showed that his PMNL gave only marginal nitroblue tetrazolium reduction and exhibited a markedly reduced bactericidal activity; his serum immunoglobulins and complement factors, serum opsonin function, and *in vitro* lymphocyte reactivity were all normal.

The present investigations occurred while both patients were apparently healthy and disclosed reduced bactericidal activity and normal ultrastructure of PMNL by electron microscopy.

PMNL Preparation. Blood was obtained from healthy donors of both sexes and from two male CGD patients during

infection- and drug-free intervals. After venipuncture, blood was allowed to drip into plastic tubes containing either EDTA (final concentration 7.7 mM) or heparin (final concentration 10 IU/ml).

Leukocytes for chemiluminescence assays were prepared from heparinized blood by dextran sedimentation and repeated saline washes (19); they were finally suspended in Hank's balanced salt solution (HBSS). Leukocytes used for the leukotriene synthesis experiments were prepared from the EDTA-treated blood as follows. The blood was centrifuged for 15 min at 200 *g* to remove the platelets. The remaining cells were mixed with an equal volume of Dextran T-500 (20 g/liter) in a sodium chloride solution (0.154 M) to remove the bulk of the erythrocytes by sedimentation. After 45 min at 4°C, the granulocyte-rich upper layer was aspirated and the cells collected by centrifugation at 200 *g* for 15 min. The cell pellet was resuspended once or twice in an ammonium chloride (0.14 M)/Tris (17 mM) solution (pH 7.4) and warmed to 37°C for 7 min to lyse residual erythrocytes. After a final centrifugation at 200 *g* for 10 min, the cell pellet was resuspended in Dulbecco's phosphate-buffered saline (pH 7.4) to the desired concentration. This purification yielded a cell suspension that contained ~90% granulocytes.

Arachidonic acid metabolism and product purification. Cell incubations followed previously reported conditions for cell number, arachidonate and ionophore concentrations, and time of incubation (9, 20, 21). These conditions were designed to produce maximal conversion of arachidonate to oxygenated products. PMNL suspensions (25 × 10⁶ cells/ml) were warmed to 37°C for 10 min before the addition of arachidonic acid (150 μM, 1.6–2.5 μCi) and the calcium ionophore A23187 (5 μM). The cells were shaken for 10 min, after which the reactions were stopped by addition of cold methanol (1.5 vol). The incubations were left at 4°C overnight and the protein precipitate was removed by centrifugation. The reaction mixtures were acidified to pH 2.5 with hydrochloric acid (3 N) and extracted with diethyl ether as previously described (22). The ether extracts were applied to silicic acid (Silicar CC-4) columns eluted sequentially with hexane/ether (90:10, vol/vol), hexane/ether (60:40, vol/vol), and ethyl acetate. This procedure generally recovered >90% of the added radioactivity from this type of incubation. Specifically, in the data presented here, the recovery was 92.6% or better in every case. The total conversion of added substrate to leukotrienes was similar to that reported in previous studies (e.g., 20).

The leukotrienes, contained in the ethyl acetate fraction, were purified by reverse-phase high performance liquid chromatography (HPLC) on a Polygosil 60-10 C₁₈ column (500 mm × 10 mm) eluted with methanol/water/acetic acid (75:25:0.01, vol/vol). The fractions eluting with the same retention value as standard compounds were collected, pooled, and evaporated to dryness. The material was dissolved in methanol (0.1 ml) and esterified with an excess of ethereal diazomethane. These solvents were removed after 5 min at room temperature and the sample was dissolved in a small volume of the straight-phase HPLC mobile phase, hexane/isopropanol/acetic acid (95:5:0.01, vol/vol) and injected onto a Nucleosil 50-5 column (250 mm × 4 mm). The solvent flow was 1 ml/min and the eluent was monitored by a UV detector set at 270 nm.

The monohydroxy acid metabolites of arachidonic acid were recovered in the hexane/ether (60:40, vol/vol) fraction and separated on reverse-phase HPLC as above. The eluent was monitored at 232 nm for these compounds.

All HPLC identifications were based on co-chromatography with standard material.

Chemiluminescence assay. PMNL chemiluminescence was determined as reported in reference 23. Briefly, PMNL (1.5×10^6 cells/ml) were suspended in HBSS with luminol (0.17 mM) and human serum albumin (10 mg/ml). The cells were preincubated at 37°C for 5 min. During this period, background light emission was monitored in a luminometer (LKB Instruments, Inc., Stockholm) and used to correct stimulated values for background. After this preincubation, stimulating agents were added and chemiluminescence was measured continuously for 1 min and periodically through 15 min. The results were expressed as maximum light emission less background in millivolts. None of the agents tested produced luminescence in the complete system with PMNL omitted.

RESULTS

PMNL arachidonic acid metabolism. PMNL were isolated from two CGD patients on several occasions. The cells were incubated with arachidonic acid and A23187 as detailed above and the leukotrienes were extracted and purified by silicic acid column chromatography. Nearly identical amounts of the extracted radioactivity were recovered in the leukotriene fraction from both control (4.8%) and patient (4.7%) leukocyte incubations. The leukotrienes were further purified by HPLC. The final analytical step was separation of the purified leukotriene methyl esters by straight-phase HPLC. The production of four major arachidonic acid metabolites was confirmed. PMNL derived from the CGD patients were capable of synthesizing LTB₄, 6-*trans*-LTB₄, 12-*epi*-6-*trans*-LTB₄, and 5(S),12(S)-dihydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid (5(S),12(S)-DHETE) (Fig. 1). These experiments lead us to suggest that there are no differences in the quantities of these dihydroxy acids produced by CGD or normal PMNL.

The monohydroxy acids derived from arachidonate were also analyzed. There were no qualitative differences between CGD and normal PMNL products. In addition, similar levels of 5-, 12-, and 15-hydroxy-eicosatetraenoic acid were synthesized by both patient and control leukocyte preparations.

Chemiluminescence. Leukocytes obtained from a group of normal volunteers produced a distinct burst of chemiluminescence when challenged with either formyl-methionyl-leucyl-phenylalanine or LTB₄ (Table I). However, PMNL of both CGD patients failed to respond when stimulated under identical conditions.

DISCUSSION

The clinical diagnosis of chronic granulomatous disease in both patients was confirmed biochemically. PMNL from the patients did not produce chemilu-

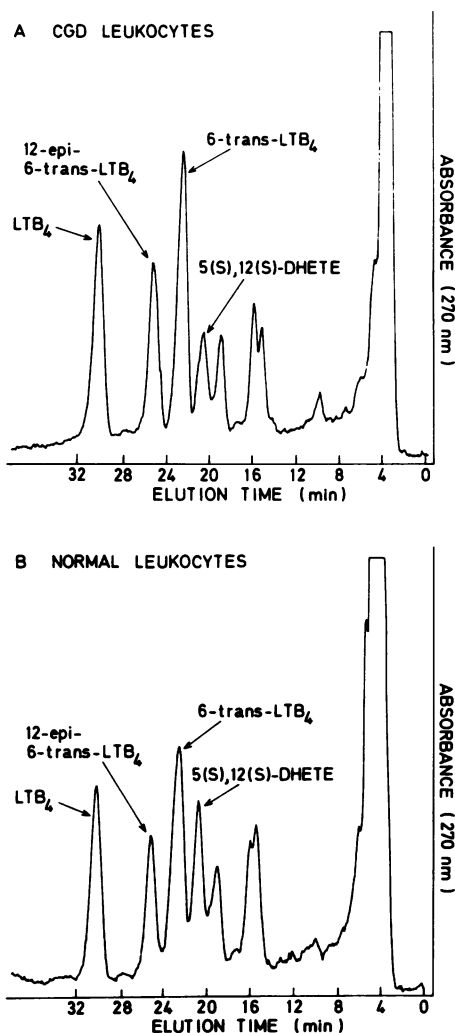


FIGURE 1 Separation of leukotriene methyl esters on straight-phase HPLC. PMNL from two CGD patients and controls were incubated with arachidonic acid (150 μ M) and A23187 (5 μ M) for 10 min at 37°C. The reaction was stopped with cold methanol. The incubation mixture was acidified and extracted with diethyl ether. The lipids were fractionated on silicic acid columns and the most polar material was subjected to reverse-phase HPLC. The main peaks of material with UV light absorption at 270 nm were pooled, converted to methyl esters, and subjected to straight-phase HPLC on a Nucleosil 50-5 column eluted with hexane/isopropanol/acetic acid (95:5:0.01, vol/vol) at a flow rate of 1 ml/min. The eluent was monitored by a UV detector set at 270 nm. (A) CGD Patient 1 leukocytes. (B) Normal leukocytes. This pattern typifies the results obtained from both patients and on several occasions.

minescence after appropriate stimulation. This indicates that active oxygen species, including superoxide anion, were not formed.

TABLE I
Chemiluminescence of Normal vs. CGD PMNL

	Stimulus	
	Formyl-methionyl-leucyl-phenylalanine (100 nM)	LTB ₄ (100 nM)
Patient 1	0.5±0.05 mV*	0 mV*
Patient 2	1.0±0.14 mV†	0 mV†
Normal	101.0±27.0 mV‡	8.3±3.5 mV§

PMNL (1.5×10^6 cells/ml) isolated from normal individuals and two CGD patients were mixed with luminol (0.17 mM) and human serum albumin (10 mg/ml) in HBSS. The cells were preincubated for 5 min at 37°C before the addition of the stated stimulating agent. Maximum light emission (in millivolts) during the following 15 min less background emission was calculated as chemiluminescence.

* Quadruplicate determinations on two occasions.

† Triplicate determinations on one occasion.

‡ Duplicate determinations: $n = 10$.

In contrast to the deficient leukocyte respiratory burst, PMNL from both patients were able to synthesize the same range and level of mono- and dihydroxy-arachidonic acid metabolites as control cells. This includes the biologically active leukotriene, LTB₄, which activates PMNL in vitro.

These studies allow a clear separation of leukotriene biosynthesis from leukocyte-generated active oxygen species. The presence of superoxide anion and its consequent oxidants is not a prerequisite to leukotriene formation. Furthermore, the oxidative metabolism of arachidonate via hydroperoxide intermediates does not contribute significantly to the formation of chemiluminescent oxygen species.

Finally, the inadequacy of CGD neutrophil bactericidal activity is not due to a failure to oxidize arachidonic acid to LTB₄. The possibility that the defect of this disease is related to the cellular response to leukotrienes rather than leukotriene synthesis is currently under investigation.

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