Relative Contribution of Leukotriene B_4 to the Neutrophil Chemotactic Activity Produced by the Resident Human Alveolar Macrophage

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Abstract

Human alveolar macrophages release chemotactic activity for neutrophils, providing a role for alveolar macrophages in regulating inflammation in the lung. As alveolar macrophages produce large amounts of leukotriene B_4 (LTB_4), a chemotactically active lipoxygenase product of arachidonic acid, we investigated the contribution of LTB_4 to the total neutrophil chemotactic activity produced by these cells. Normal human alveolar macrophages were recovered by bronchoalveolar lavage from healthy volunteers and incubated either with the calcium ionophore A23187 for 1 h, or with opsonized zymosan particles or latex beads for 3 h. Nordihydroguaretic acid (NDGA), a relatively specific lipoxygenase inhibitor, blocked the release of neutrophil chemotactic activity after all three stimuli in a dose-dependent manner. This correlated with blockade of LTB_4 production as measured by high performance liquid chromatography using freshly isolated alveolar macrophages, as well as blockade of $[^3H]LTB_4$ production by macrophages prelabeled with $[^3H]$arachidonate. Molecular sieve chromatography using Sephadex G-50 confirmed that essentially all of the chemotactic activity in the stimulated macrophage supernatants co-eluted with authentic $[^3H]LTB_4$, and that NDGA completely blocked the chemotactic activity in the eluting fractions. Readdition of authentic LTB_4 (1×10^{-7} M) to the NDGA-blocked macrophage supernatants restored the chemotactic activity in the supernatants. The macrophage supernatants did not contain platelet-activating factor–like activity, as measured by the stimulation of $[^3H]$serotonin release from rabbit platelets, and by high performance liquid chromatography. NDGA did not change the protein-secretion profiles of fresh alveolar macrophages, or of macrophages prelabeled with $[^{35}S]$methionine. The complement (C) components $C5a_{desarg}$ were not detected in any of the supernatants by radioimmunoassay. Concentration of the supernatants by positive pressure filtration (5,000-D membrane) did not augment chemotactic activity in the stimulated supernatants or uncover chemotactic activity in the NDGA-blocked supernatants. As with the 3-h studies, when alveolar macrophages were incubated overnight with opsonized zymosan, all of the increase in chemotactic activity could also be blocked by NDGA. These data indicate that LTB_4 is the predominant neutrophil chemotactic factor secreted by the normal resident human alveolar macrophage in response to two major types of stimuli, calcium fluxes across the cell membrane and the phagocytosis of opsonized particulates.

Introduction

The alveolar macrophage (AM)[1] plays an important role in the defense of the conducting airways and alveolar membrane against particulates that elude the physical barriers of the upper airway. It is unlikely, however, that AM function as solo defenders of the alveolar spaces, because compared with other phagocytes, AM migrate sluggishly in response to chemotactic stimuli, and kill microorganisms slowly. Rather, a more important function of the resident AM may be the initiation and amplification of inflammatory responses in the lung via the secretion of chemotaxins that recruit effector cells to the lung (1–3).

The first demonstration that AM release chemotactic activity for blood neutrophils followed the observation that repeated bronchoalveolar lavage of primate lungs led to the accumulation of neutrophils in bronchoalveolar lavage fluid (1). The bronchoalveolar lavage fluid contained two identifiable chemotactic factors, the larger of which was thought to be C5a, as its apparent molecular weight was 15,000 and its activity was inhibitable by anti–C5 antibodies. The smaller factor had an apparent molecular weight of ~ 5,000 and was not inhibited by anti–C5 antibodies. It was detected in both concentrated lavage fluid and concentrated supernatants of AM monolayers incubated in vitro. This factor was released from primate AM stimulated by adherence to glass or by phagocytosis of opsonized, heat-killed staphylococci (1).

Human AM have also been shown to produce neutrophil chemotactic activity but there is conflicting evidence about the nature of the chemotactic substance(s) (2, 3). Merrill and associates (2) described two chemotactic substances in concentrated supernatants of human AM from nonsmoking subjects. The larger factor (apparent molecular weight of 10,000) was trypsin sensitive and appeared to be distinct from C5 or C3a, as its apparent activity was not inhibited by antisera to these complement (C) fragments. Isoelectric focusing of the concentrated larger factor revealed five separate fractions, of which only one had significant chemotactic activity (pI = 5.0). The smaller chemotactic factor (apparent molecular weight < 1,000) was not characterized. Hunninghake and associates

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1. *Abbreviations used in this paper:* AM, alveolar macrophage; DEC, diethylcarbamazine; HETE, monohydroxyeicosatetraenoic acid; HPF, high-power field; LB, opsonized latex beads; LTB_4, LTC_4, LTD_4, LTE_4, and LTF_4, leukotrienes B_4, C_4, D_4, E_4, and F_4, respectively; NDGA, nordihydroguaretic acid; OZ, opsonized zymosan particles; PAF, platelet-activating factor; PMN, polymorphonuclear neutrophilic leukocytes.

(3) found that human AM stimulated with immune complexes or particulates produce a low molecular weight (400–600) chemotactic factor that was insensitive to a variety of proteases. It was 80% extractable into lipid solvents, which suggested that this factor might be lipid derived, but analysis by gas chromatography and mass spectroscopy suggested that it was not a monohydroxyeicosatetraenoic acid (HETE). A similar low molecular weight chemotactic factor also is produced by guinea pig AM (4, 5).

One of the most active lipid-derived chemotactic substances is leukotriene B_4 (LTB_4), a 5-lipoxygenase product of arachidonic acid metabolism produced by polymorphonuclear and mononuclear phagocytes (6). Arachidonic acid constitutes nearly 20% of the membrane lipid of rabbit AM (7), and human blood monocytes (8) and human AM release large amounts of LTB_4 after stimulation with either soluble or particulate stimuli (9–11). Evidence from rabbit AM suggests that LTB_4 may account for a large part of the chemotactic activity produced by these cells, as blockade of the lipoxygenase pathway abolishes the release of neutrophil chemotactic activity after particulate stimuli (12).

As LTB_4 has many features in common with the previously described low molecular weight neutrophil chemotactic factor produced by AM, the goal of this study was to determine the relative contribution of LTB_4 to the neutrophil chemotactic activity produced by the resident human AM.

Methods

Special reagents. Synthetic LTB_4, 20-COOH-LTB_4, 20-OH-LTB_4, LTC_4, LTD_4, and LTE_4, and 5-(*S*),12-(*S*)-di-HETE were kindly provided by Dr. J. Rokach, Merck Frosst Laboratories, Pointe-Claire, Dorval, Quebec. The HETE standards (5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE, and 15-HETE) were provided by Dr. W. C. Hubbard, National Institutes of Health, Bethesda, MD. 5-(*S*),12-(*R*)-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid (abbreviated 5-(*S*),12-(*R*)-6-*trans*-LTB_4) and 5-(*S*),12-(*S*)-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid (abbreviated 5-(*S*),12-(*S*)-6-*trans*-LTB_4) were purified from neutrophils stimulated by the calcium ionophore A23187 as previously described (13). [5,6,8,9,11,12,14,15-^{3}H(*N*)]-Arachidonic acid (60–100 Ci/mmol), 14,15-[^{3}H(*N*)]LTB_4 (30–60 Ci/mmol), 1-*O*-(alkyl-1,2-^{3}H), alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (platelet-activating factor [PAF]) (30–60 Ci/mmol), and [^{3}H]-acetic acid, sodium salt (500 mCi/mmol) were obtained from New England Nuclear, Boston, MA. The calcium ionophore A23187 was obtained from Calbiochem-Behring Corp. La Jolla, CA.

Cell recovery. AM were recovered as previously described from the lungs of 23 healthy volunteers who gave written informed consent (11). All were free of respiratory diseases and none used cigarettes or medications of any kind. Briefly, subsegments of the right middle lobe and the lingula each were lavaged five times with 50-ml aliquots of sterile, pyrogen-free 0.89% NaCl (total volume, 250 ml in each site) using a flexible fiberoptic Olympus BF-3 bronchoscope. The procedure was well tolerated, as determined by a direct questioning of each volunteer by telephone during the evening after the procedure. The lavage fluid was recovered with gentle suction, pooled, filtered through moist gauze to remove mucus, and centrifuged at 600 *g* for 15 min at 4°C to pellet the cells. The supernatants were decanted and the cells washed twice in Hanks' balanced salt solution (HBSS, Gibco, Grand Island, NY). Total cell counts were performed in a hemocytometer and differential cell counts were performed on cytocentrifuge preparations stained with Dif-Quik (Scientific Products, McGaw Park, IN). The mean cell recoveries were (all values are mean±SD): total white cells, $17.4\pm9.8 \times 10^6$; macrophages, 93.0±8.5%; neutrophils, 1.8±0.5%; and lymphocytes, 5.2±8.7%. The macrophage viability averaged 89.2±3.6%.

Preparation of stimulated macrophage supernatants. To stimulate the release of neutrophil chemotactic activity, the macrophages were incubated at 2.5×10^6/ml in either HBSS or Tyrode's buffer in rotating tubes (to minimize adherence) at 37°C in 5% CO_2/air for 1 h with the calcium ionophore A23187 (10 μg/ml) (Calbiochem-Behring Corp.), or for 3 h with one of two different particulate stimuli, opsonized zymosan particles (OZ) (2×10^8/ml) (Sigma Chemical Co., St. Louis, MO), or opsonized latex beads (LB) (2×10^8/ml) (Sigma Chemical Co.). The OZ were boiled and washed extensively before use. To facilitate phagocytosis, the particulate stimuli were preopsonized by incubation for 1 h at 37°C with 50% fresh human serum. They were then washed extensively and stored frozen at −70°C. In some experiments, nordihydroguaretic acid (NDGA), or diethylcarbamazine (DEC) were added at least 5 min before adding the stimuli to block the lipoxygenase pathway of archidonic acid metabolism. To confirm the findings with opsonized zymosan and latex beads, additional experiments were performed using opsonized *Staphylococcus aureus,* 502a. The *S. aureus* were grown overnight in trypticase soy broth, washed, resuspended to an optical density of 0.2 at 540 nm, and added to human AM in Tyrode's buffer containing 5% autologous human serum as an opsonin. To test the effects of a longer incubation period in a more complete medium, we performed additional experiments in which AM were incubated overnight in tissue culture media (RPMI 1640, Gibco) containing 0.5% bovine serum albumin with or without OZ and NDGA.

After each incubation, cell viability was measured by trypan blue exclusion and, for particulate stimuli, the number of cells with cell-associated particles was counted by light microscopy. The cell suspensions were centrifuged at 600 *g* for 15 min at 4°C and the supernatants were decanted, filtered (0.22-μm filters, Millipore Corp., Bedford, MA), and stored at −70°C until assayed. The neutrophil chemotactic activity in each supernatant was measured within 3 wk. In some experiments the supernatants were concentrated before assay by positive pressure filtration under nitrogen at 4°C using a membrane with a 5,000 mol wt limit (YM-5; Amicon Corp., Danvers, MA).

Chemotaxis assay. The chemotaxis of normal human peripheral blood neutrophils toward the AM supernatants was measured in microchemotaxis chambers using nitrocellulose membranes with 3.0-μm–sized pores (11, 14). Neutrophils were obtained from the peripheral blood of healthy volunteers by Ficoll-Hypaque density gradient centrifugation, followed by dextran sedimentation and hypotonic lysis of residual erythrocytes (15). A different donor was used for each separate experiment. The neutrophils were washed and resuspended in Gey's balanced salt solution (Gibco) at a final concentration of 1.0×10^6/ml. 25 μl of the undiluted macrophage supernatant was added to each lower well of the microchemotaxis chambers and 50 μl of cells were added to each upper well. After incubation for 2 h in 5% CO_2/air, the membranes were removed, stained with Dif-Quik, and mounted on microscope slides. Chemotaxis was measured with a 1.0-mm^2 eyepiece grid as the total number of cells that migrated through each filter (within the grid) in 10 consecutive light microscopic fields (× 540). In each experiment, the chemotactic activity in each supernatant was tested in quadruplicate wells and the results were averaged.

Molecular sieve chromatography. To determine the apparent molecular weight of the chemotactic activity in the AM supernatants, the supernatants were applied to a 1 × 51-cm Sephadex column (G-50 fine; Pharmacia Fine Chemicals; Piscataway, NJ) and eluted with 0.05 M NH_4HCO_3 buffer, pH 7.2, at a flow rate of 8.2 ml/h at 4°C. The optical density of the eluate was measured continuously at 280 nm and 2.0-ml fractions were collected and assayed for neutrophil chemotactic activity. The column was calibrated with protein standards of known molecular weight (Pharmacia Fine Chemicals), and with synthetic [^{3}H]LTB_4 and ^{3}H-PAF standards.

Arachidonic acid lipoxygenase products. To measure the release of radiolabeled arachidonic acid products after each type of stimulus, AM were incubated overnight (20 h) in RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (40 μg/ml), and [^{3}H]arachidonic acid,

0.5 μCi/10^6 macrophages. After labeling, the cells were washed three times to remove free [^{3}H]arachidonic acid, resuspended in either HBSS or Tyrode's solution at 2.5 × 10^6 viable cells/ml, and stimulated as described. The uptake of [^{3}H]arachidonic acid was determined using scintillation counting by comparing the number of counts in the cell pellets after lysis with 0.1% Triton-X (New England Nuclear) with the total number of counts in each tube (supernatant plus pellet). The release of radiolabeled arachidonic acid products was measured by comparing the number of counts in each supernatant with the total number of counts in each tube (supernatant plus pellet).

The lipoxygenase products of arachidonic acid were measured in each supernatant by high performance liquid chromatography (HPLC) as previously described (11), Briefly, the supernatants were prepared for HPLC by initial solid-phase extraction using octadecyl Baker-10 columns (J. T. Baker Chemical Co., Phillipsburg, NJ) followed by evaporation to dryness and reconstitution in methanol. After centrifugation at 3,000 *g* for 5 min at 4°C, the samples underwent reverse-phase HPLC on a 4.6 × 250-mm Ultrasphere ODS C_{18} column (5 μm particle size, Beckman Instruments, Inc., Berkeley, CA) using methanol/water/acetic acid (75:25:0.01, vol/vol/vol), pH 4.7, at a flow rate of 1 ml/min (HPLC condition I). Peaks at 280 and 235 nm that co-eluted with authentic leukotriene and HETE standards were collected and rechromatographed on a 4.6 × 75-mm Ultrasphere ODS C_{18} column (3 μm particle size, Beckman Instruments, Inc.) using the same solvent at a flow rate of 1.6 ml/min (HPLC condition II). The retention times (average range) of the lipoxygenase standards using HPLC conditions I and II, respectively, were as follows: 20-COOH-LTB_4, 3.8–4.0 min, 0.6–0.7 min; 20-OH-LTB_4, 3.9–4.3 min, 0.7–0.9 min; 5-(*S*), 15-(*S*)-diHETE, 8.3–9.4 min, 1.8–2.1 min; 8-(*S*), 15-(*S*)-diHETE, 8.7–9.4 min, 1.2–1.3 min; 5-(*S*), 12-(*R*)-6-*trans*-LTB_4, 9.1–10.3 min, 1.8–1.9 min; 5-(*S*), 12-(*S*)-6-*trans*-LTB_4, 10.6–11.3 min, 2.0–2.1 min; LTB_4 and 5-(*S*), 12-(*S*)-diHETE, 12.5–14.1 min, 2.3–2.5 min; LTD_4, 16.8–18.1 min, 3.0–3.3 min; LTC_4, 21.7–24.4 min, 5.1–5.47 min; 15-HETE, 31.8–32.6 min, 6.1–6.3 min; 11-HETE, 34.8–36.2 min, 6.4–6.7 min; 8-HETE and 9-HETE, 36.1–37.7 min, 6.8–7.2 min; 12-HETE, 37.4–39.9 min; 7.6–8.0 min; and 5-HETE, 43.6–47.1 min, 8.0–8.4 min. As LTB_4 and 5-(*S*), 12-(*S*) diHETE standards co-eluted by reverse-phase HPLC, the peaks collected at the retention time of these compounds were converted to their methyl esters with excess diazomethane in diethyl ether and resolved by straight-phase HPLC on a μPorasil column (3.9 × 300 mm, 10 μm particle size, Waters Assoc., Milford, MA), as previously described (9). Using hexane/isopropanol/acetic acid (95:5:0.01, vol/vol/vol) at a flow rate of 1 ml/min (HPLC condition III), the retention times of 5-(*S*), 12-(*S*)-diHETE and LTB_4 methyl ester standards were 16.7–18.4 min and 23.8–26.0 min, respectively. The ultraviolet spectra of the rechromatographed peaks were measured using a recording spectrophotometer (model 219; Cary Instruments, Monrovia, CA) and the amount of each compound was calculated using the extinction coefficient for each identified arachidonate metabolite as previously described (12).

In some experiments, LTB_4 in AM supernatants was assayed by the competitive inhibition of the binding of [^{3}H]LTB_4 to anti-LTB_4 antibodies. Rabbit anti-LTB_4 serum was the generous gift of Drs. Robert W. Egan and John L. Humes, Merck Institute for Therapeutic Research (Rahway, NJ). The serologic specificity of the anti-LTB_4 antiserum, determined as the amount in picomoles of each ligand required for 50% inhibition of [^{3}H]LTB_4 is as follows, with the percentage crossreactivity given in parentheses (16): 5-(*S*), 12-(*R*)-LTB_4, 0.71 pmol (100%); 5-(*S*), 12-(*R*)-6-*trans*-LTB_4, 12 pmol (6%); 5-(*S*), 12-(*S*)-6-*trans*-LTB_4, > 1,000 pmol (0%); 5-(*S*), 12-(*S*)-LTB_4, 290 pmol (0.2%); 5-(*S*), 12-(*S*)-6,10-*trans*-8,14-*cis*-diHETE, > 10 pmol (< 7%); 20-OH-LTB_4, 35 pmol (2.0%); 20-COOH-LTB_4, > 1,000 pmol (0%); 5-HETE, 330 pmol (0.2%); LTC_4, 3,100 pmol (0.02%); LTD_4, 1,500 pmol (0.04%); LTE_4, 470 pmol (0.15%); and LTF_4, > 3,100 pmol (0%). Each assay was performed in duplicate according to published procedures (16).

PAF. The presence of PAF activity in the macrophage supernatants was measured using the release of [^{3}H]serotonin from rabbits platelets as a biologic endpoint (17, 18), and also by HPLC (19). Platelets were recovered from citrated rabbit blood by slow speed centrifugation (150 *g*) for 15 min, then incubated with [^{3}H]serotonin (0.5 μCi/10^8 platelets, New England Nuclear) in Tyrode's buffer containing 0.25% gelatin. They were washed three times to remove the free label, then resuspended at a concentration of 1.0 × 10^8 platelets/ml in Tyrode's buffer containing 0.25% gelatin. Platelets (1 × 10^8) and macrophage supernatant (100 μl) were combined in a total volume of 500 μl and incubated for 15 min at 21°C. The reaction tubes then were centrifuged and the platelets lysed in 0.1% Triton-X for 15 min. The release of [^{3}H]serotonin was determined by dividing the radioactivity in the supernatants by the total radioactivity in the tube (supernatant and lysed pellet), and expressed as percentage release. In each assay, a standard curve using authentic PAF (Calbiochem-Behring Corp.) was included for comparison. In some experiments, authentic PAF was added at known concentrations to macrophage supernatants to ensure that the supernatants did not contain a factor that blocked the detection of PAF activity. Only supernatants of particulate-stimulated macrophages were assayed, because control experiments indicated that A23187 directly stimulated [^{3}H]serotonin release from rabbit platelets.

To assess the presence of PAF using HPLC (19), AM were incubated overnight with [^{3}H]acetate to label membrane acetate pools. The macrophages were washed twice to remove free label and then stimulated with A23187 in the presence or absence of NDGA. Lipids were extracted from the supernatants by the method of Bligh and Dyer (20) using chloroform/methanol/water (1:1:0.9, vol/vol/vol). The lipid extracts were dried under nitrogen, resuspended in isopropanol/hexane (1:1, vol/vol) and underwent HPLC on a 5-μm Ultrasphere-Si column (4.6 × 250 mm) with monitoring at 205 nm for isolation of PAF according to the method of Blank and Synder (21). Samples were eluted at a flow rate of 2 ml/min with a starting solvent of 96% isopropanol/hexane (1:1, vol/vol) and 4% H_2O changing to 8% H_2O in a linear gradient over a 15-min period from the time of injection (HPLC condition IV). The radioactivity of the eluting peaks was measured with a radioactive flow detector (Flo One, model HP; Radiomatic Instruments & Chemical Co., Inc.) using Radiomatic Flo-Scint II at a ratio of 3:1 (scintillant/HPLC solvent). In agreement with previous findings (21), the ^{3}H-PAF standard (retention time range, 30.1–32.4 min) was completely resolved from the following phospholipids (at the indicated retention times); phosphatidylglycerol (6.7–7.3 min); phosphatidylethanolamine and phosphatidylinositol (9.2–10.7 min); phosphatidic acid (12.9–14.1 min); phosphatidylserine (16.5–18.3 min); phosphatidylcholine (20.9–23.0 min); sphingomyelin (22.6–25.6 min); acylacetylglycerophosphocholine (32.6–35.8 min); and lysophosphatidylcholine (34.6–38.1 min).

Proteins. To determine whether the various stimuli might affect protein secretion by the AM and whether NDGA also might block the secretion of these proteins, we analyzed the supernatants for total protein and protein profiles. Total proteins were measured by the Lowry method (22) in an assay modified for microtiter plates (23). To measure the release of preformed proteins from the macrophages, aliquots of the supernatants were dissolved in Laemmli sample buffer (24) and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in continuous gradients (10–20 or 5–15% acrylamide) with and without β-mercaptoethanol as a reducing agent. The gels were stained with either a silver stain method (25) or with Coomassie Blue. Molecular weight standards were purchased from Pharmacia Fine Chemicals.

To measure the release of newly synthesized proteins, human AM were incubated in methionine-deficient Dulbecco's modified Eagle's medium (Gibco) supplemented with L-glutamine (584 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and bovine serum albumin (1 mg/ml), for 1 h at 37°C in 5% CO_2/air. The cells were sedimented at 200 *g* and resuspended in the same medium supplemented with [^{35}S]methionine (New England Nuclear) at a final concentration of 50 μCi/ml. The cells were incubated in this medium for 16 h with gentle agitation. After labeling, the cells were washed, resuspended in HBSS, and stimulated as described above. These supernatants were

analyzed by SDS-PAGE as described, and the gels were stained with Coomassie Blue to idenify the position of the molecular weight standards, then impregnated with Enhance (New England Nuclear), dried, placed on Kodak X-Omat AR film, and exposed at −70°C for 14 d.

To determine whether the chemotactically active C fragments C5a or $C5a_{desarg}$ might account for a portion of the chemotactic activity in the supernatants, we tested each supernatant for these products by radioimmunoassay as previously described (26) using a commercially available antibody (Upjohn Co., Kalamazoo, MI). This method can detect 0.1 ng/ml of C5a and $C5a_{desarg}$ and the antibody does not crossreact with either C3 or C4.

Statistics. Differences in chemotactic activity and protein concentrations between stimulated and unstimulated samples were analyzed by one-way analysis of variance and by Student's *t* test (27). A *P* value of 0.05 or less was accepted as significant.

Results

Release of chemotactic activity. Stimulation of normal human AM with the calcium ionophore A23187, OZ, and LB caused the release of neutrophil chemotactic activity into the macrophage supernatants (Fig. 1). The ionophore caused the greatest release of chemotactic activity (~ 250 polymorphonuclear neutrophilic leukocytes [PMN]/10 high-power fields [HPF]), and this was nearly twofold greater than that induced by either OZ or LB. OZ and LB caused the release of equivalent amounts of chemotactic activity, despite the fact that the macrophages appeared to ingest approximately five times more LB than OZ, by light microscopy.

The addition of NDGA (10^{-5}–10^{-4} M) inhibited the release of neutrophil chemotactic activity in a dose-dependent fashion from both ionophore- and particle-stimulated macrophages (Fig. 1). The NDGA did not affect macrophage viability as measured by trypan blue exclusion or the number of cells that ingested particles. Similar results were obtained using *S. aureus* as the particulate stimulus. As with OZ and LB, NDGA (10^{-4} M) inhibited 100% of the neutrophil chemotactic activity in the supernatants of AM incubated with *S. aureus* ($P < 0.05$).

These results were not unique to NDGA, as DEC (10^{-3} M) also inhibited the generation of neutrophil chemotactic activity in response to OZ by > 90%. Control experiments established that NDGA and DEC at the concentrations used did not inhibit neutrophil chemotaxis toward either LTB_4 (10^{-7} M) or zymosan-activated serum.

Molecular sieve chromatography. To estimate the apparent molecular weight of the neutrophil chemotactic activity, and to determine whether any chemotactic activity was associated with the protein-containing fractions of the supernatants, particularly those in the 10–15 kD range as had been previously suggested (2), we performed molecular sieve chromatography using Sephadex G-50 to analyze the supernatants of AM from three separate volunteers. The chemotactic activity in the ionophore- and particulate-stimulated supernatants eluted in late fractions (tubes 34 to 38) with a retention time corresponding to that of synthetic [^{3}H]LTB_4 (Figs. 2 *A* and 3 *A*). No chemotactic activity was present in the protein-containing fractions of either A23187 or zymosan-stimulated macrophages. Authentic ^{3}H-PAF consistently eluted 3 to 4 tubes earlier than either [^{3}H]LTB_4 or the chemotactic activity. When [^{3}H]LTB_4 and ^{3}H-PAF where applied simultaneously to the column, twin peaks of radioactivity were observed, with ^{3}H-PAF eluting four tubes earlier than [^{3}H]LTB_4, indicating that the retention time of the chemotactic activity is similar to that of LTB_4 and distinct from that of PAF. In the supernatants of the NDGA-treated macrophages, the chemotactic activity in the late fractions (tubes 34 to 38) was almost completely abolished for both the A23187 and the zymosan-stimulated macrophages (Figs. 2 *B* and 3 *B*).

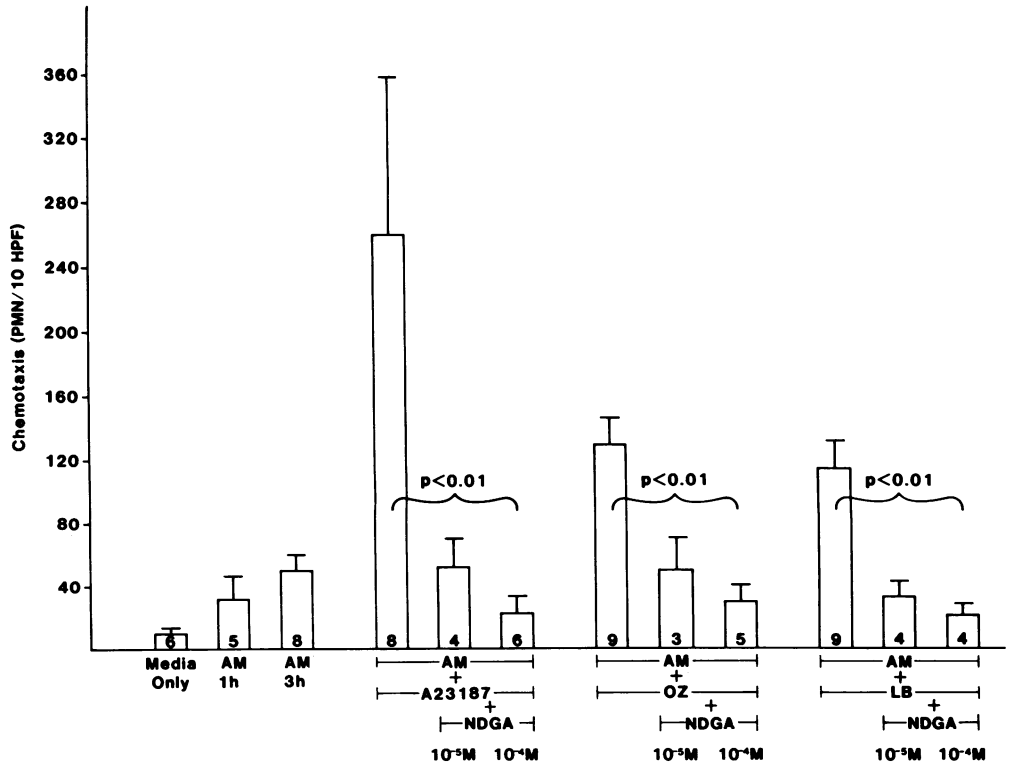


Figure 1. The effect of NDGA on AM derived neutrophil chemotactic activity (mean±SE). AM were incubated for 1 h with A23187 (10 μg/ml), or for 3 h with OZ (2×10^8 particles/ml) or LB (2×10^8 beads/ml). In some experiments, the macrophages were preincubated for 15 min in NDGA at the indicated concentrations. Neutrophil chemotaxis in response to each undiluted supernatant is indicated on the vertical axis as the number of neutrophils migrating through the nitrocellulose filter in 10 high power microscopic fields. The numbers in the bars indicate the number of experiments, each using AM from a different human volunteer and peripheral blood neutrophils from different donors.

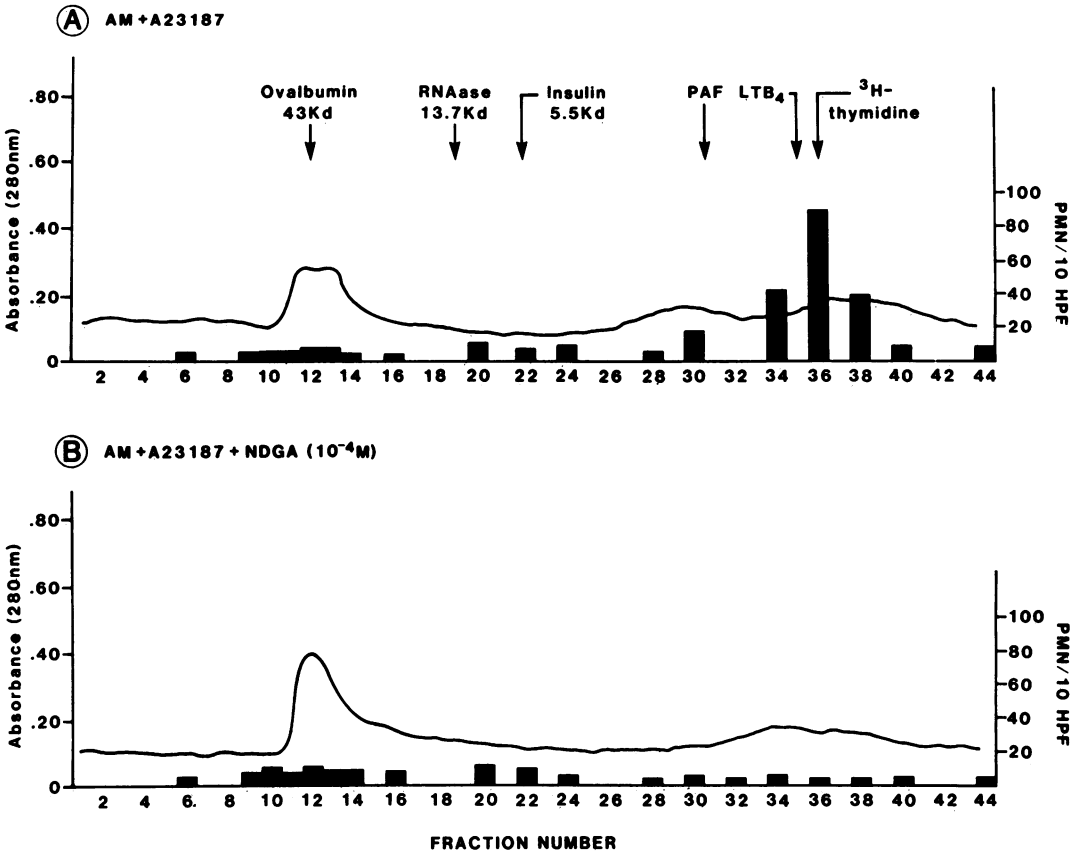


Figure 2. Molecular sieve chromatography using Sephadex G-50 of supernatants of AM stimulated with A23187 (10 μg/ml) in the absence (*A*) or presence (*B*) of NDGA (10^{-4} M). Shown across the *top* are the positions at which the various molecular weight standards eluted from the column. A 2.0 ml vol of macrophage supernatant was added to the column, which was eluted with 0.05 M $NaHCO_3$ buffer, pH 7.2, and 2.0-ml fractions were collected. The continuous line indicates the protein concentration in each eluting fraction. The vertical bars indicate the neutrophil chemotactic activity in each fraction. Similar results were obtained with the supernatants of AM from three separate volunteers.

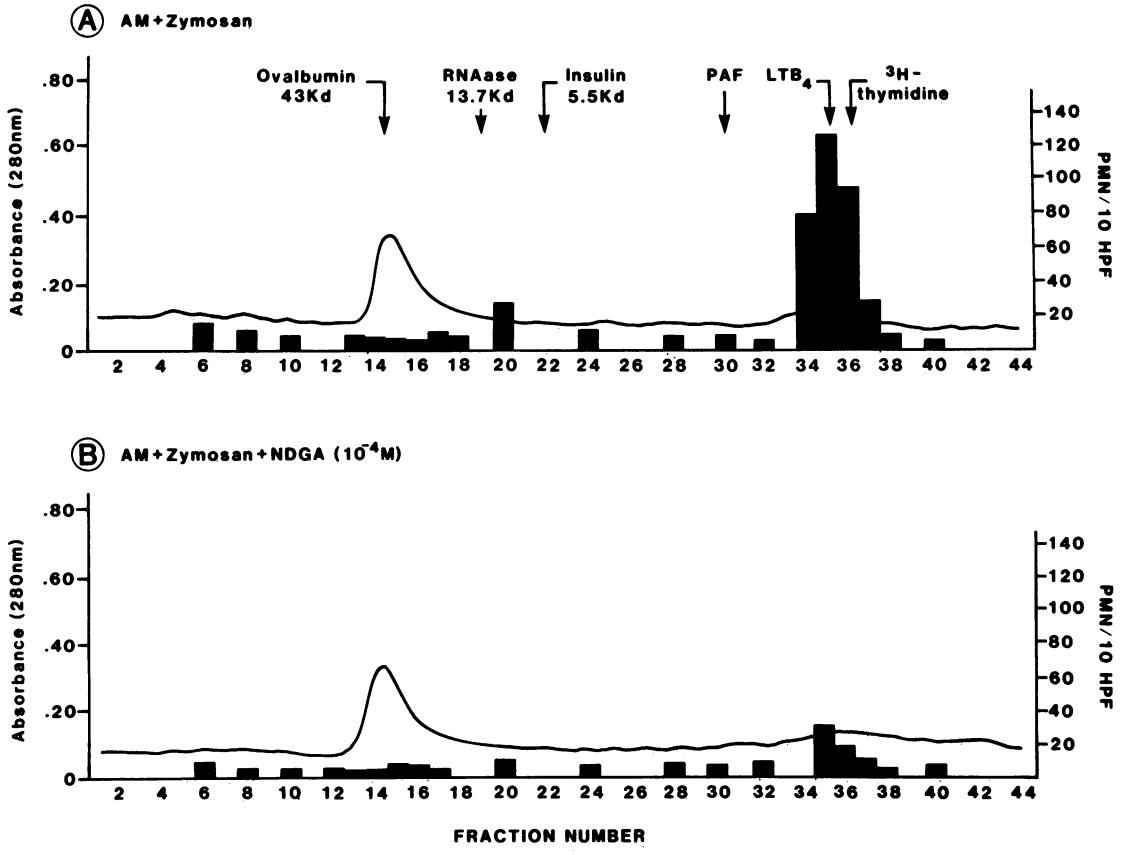


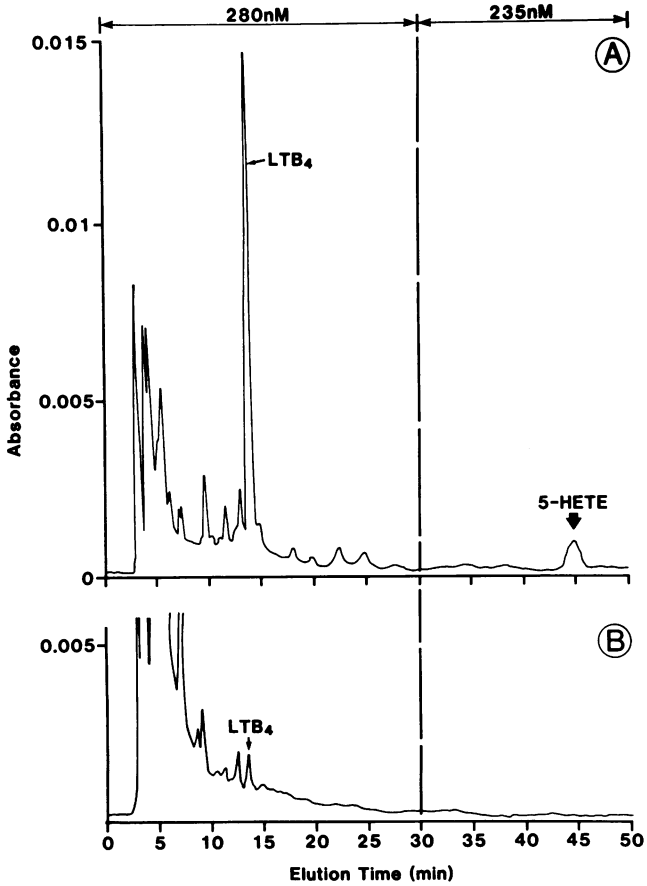
Figure 3. Molecular sieve chromatography of supernatants of AM stimulated with OZ in the absence (*A*) or presence (*B*) of NDGA. The format is the same as for Fig. 2.

Arachidonic acid products. The presence of neutrophil chemotactic activity in each supernatant correlated with the release of arachidonic acid products into the supernatants, as measured by HPLC (Fig. 4). The predominant product detected by HPLC in each of the stimulated supernatants was LTB_4. In five separate experiments, human AM incubated with [^{3}H]arachidonic acid for 20 h incorporated (64.2±10.4% (mean±SD) of the added radiolabel into the cell membranes. After stimulation with A23187, 28.9±7.8% of the incorporated activity was released into the supernatant; 17.2±3.4% was released after phagocytosis of OZ. Unstimulated macrophages released 9.2±4.7% of the incorporated arachidonate. The ionophore caused the greatest release of LTB_4 (23.5±5.8 ng/10^6 AM), in agreement with our earlier findings (11). Smaller amounts were detected after particulate stimulation (OZ, 7.9±0.4 ng/10^6 cells; LB, 9.2±1.7 ng/10^6 cells).

The NDGA blocked the release of [^{3}H]arachidonic acid lipoxygenase products from AM stimulated with either A23187, OZ, or LB (Fig. 4). At lower concentrations (10^{-5} M), NDGA blocked predominantly LTB_4 and HETE production, whereas at higher concentrations (10^{-4} M), NDGA also partially blocked the early eluting peaks (4–8) min) that represent predominantly cyclooxygenase products. The detection of LTB_4 by HPLC was not affected by the presence of NDGA. In two separate experiments using AM from two different human subjects, NDGA (10^{-4} M) also significantly reduced the production of LTB_4 by *S. aureus*–treated AM, as detected by radioimmunoassay (18.3±8.0 ng LTB_4/10^6 cells without NDGA vs. 0.2±0.1 ng LTB_4/10^6 cells with NDGA). For all of the macrophage stimuli, the reduction in neutrophil chemotactic activity in the presence of NDGA paralleled the reduction in the amounts of LTB_4 detected in the supernatants.

To determine whether the reduction in chemotactic activity in the presence of NDGA could be accounted for by the reduction in LTB_4 concentration in the supernatants, we performed add-back experiments in which known amounts of LTB_4 were added to supernatants generated in the presence of NDGA (10^{-4} M) (Table I). The addition of LTB_4 to these supernatants increased the amount of neutrophil chemotactic activity in a dose-dependent manner. At a concentration of LTB_4 that approximated that found in the original supernatants (5×10^{-7} M), the chemotactic activity of the original supernatant was almost entirely restored.

PAF. Fig. 5 shows that zymosan-stimulated and -unstimulated macrophage supernatants from three separate human volunteers failed to cause the release of significant amounts of [^{3}H]serotonin from rabbit platelets. To be certain that potential PAF activity in the macrophage supernatants was not blocked by an inhibitor in the supernatants, we added authentic PAF to each of the zymosan-stimulated and NDGA-treated



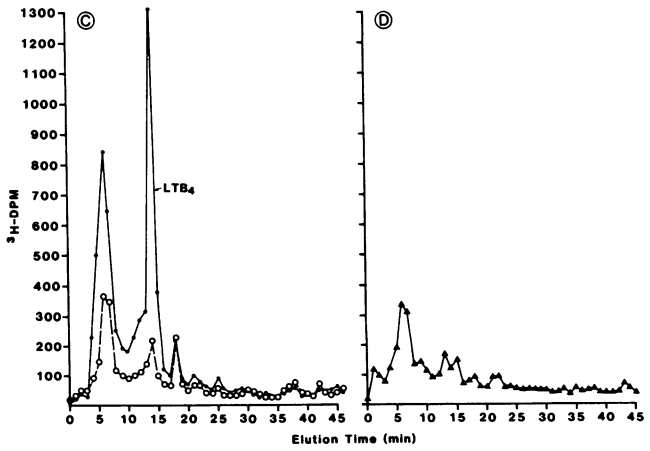


Figure 4. The effects of NDGA on the release of arachidonic acid metabolites by alveolar macrophages. (*Left*) HPLC chromatograms (HPLC condition I, see Methods) of supernatants of AM stimulated for 1 h with A23187 (10 μg/ml) in the absence (*A*) or presence (*B*) of NDGA (1×10^{-4} M). (*Right*) (*C*) HPLC profile of [^{3}H]arachidonic acid metabolites released by AM that were labeled overnight with [^{3}H]arachidonic acid, then stimulated for 3 h with OZ in the absence (●) or presence (○) of NDGA (1×10^{-4} M). (*D*) The HPLC profile of a supernatant of [^{3}H]arachidonate labeled AM incubated for 3 h in buffer alone.

Table I. Reconstitution of Neutrophil Chemotactic Activity in NDGA-blocked Macrophage Supernatants by the Addition of LTB_4

Supernatant	PMN chemotaxis
	PMN/10 HPF
LTB_4 standard (10^{-7} M)*	97.0±4.4
PBS*	14.5±1.6
AM + OZ*	134.7±13.3
AM + OZ + NDGA (10^{-4} M)*	37.0±3.0
+ LTB_4 (1×10^{-6} M)‡	119.7±1.9
+ LTB_4 (5×10^{-7} M)‡	129.3±9.0
+ LTB_4 (1×10^{-7} M)‡	100.0±4.2
+ LTB_4 (5×10^{-8} M)‡	57.3±3.1
+ LTB_4 (1×10^{-8} M)‡	51.0±2.1

The data are the mean±SE of at least three separate experiments. See text for the measurement of chemotaxis.
* Chemotactic responses to the LTB_4 standard, phosphate-buffered saline (PBS) alone, and supernatants of AM incubated with OZ for 3 h.
‡ Authentic LTB_4 was added in the concentrations indicated to supernatants of AM that had been incubated with OZ and NDGA (1×10^{-4} M).

supernatants and found that the added PAF was fully detectable (Fig. 5). Supernatants containing A23187 could not be tested by this method, as the ionophore causes platelet serotonin release in this assay. However, PAF was not detected by HPLC in supernatants obtained from [^{3}H]acetate-labeled AM that were stimulated with the calcium ionophore A23187.

Proteins. As secretory stimuli may cause the release of preformed proteins (28) in addition to membrane lipids, we investigated whether NDGA might also affect the secretion of proteins that have neutrophil chemotactic activity. The ionophore was a potent secretagogue, increasing the protein concentration in the supernatants by approximately three times, but the particulate stimuli caused little additional release of proteins into the unconcentrated supernatants (Fig. 6). Although the addition of NDGA tended to reduce the protein secretion partially for each of the stimuli tested, none of the apparent reductions in protein secretion were statistically significant when compared with the respective stimulated values.

Analysis of the supernatants of unlabeled and [^{35}S]-methionine labeled macrophages by SDS-PAGE and autofluorography showed that the types of proteins secreted were similar in the stimulated and the NDGA-blocked supernatants (Fig. 7). The NDGA did not affect the pattern of release of either preformed or metabolically labeled proteins in response to either A23187 or OZ, particularly those with a <43,000 mol wt.

To determine whether the macrophage supernatants might contain one or more chemotactically active proteins in low concentration, we incubated AM from two different volunteers with A23187 with or without added NDGA, then combined the supernatants to increase the starting volume, and concentrated them approximately five- to sixfold by positive pressure filtration at 4°C under nitrogen. The resulting fluids were tested twice for chemotactic activity and the results are shown in Table II. The concentration procedure did not augment the neutrophil chemotactic activity in the A23187-stimulated supernatant. The chemotactic activity in the protein-free filtrate was similar to that of the protein-enriched concentrate, indicating that the chemotactic activity distributes relatively evenly across this type of membrane (5,000 mol wt limit). In addition, concentrating the NGDA-blocked supernatant in a similar fashion did not increase the neutrophil chemotactic activity in that supernatant.

As macrophages have been reported to secrete complement components (29), we analyzed the stimulated macrophage supernatants for the presence of C5a and $C5a_{desarg}$ by radioimmunoassay to determine whether these proteins might account for some of the chemotactic activity in the unconcentrated supernatants. However, none of the supernatants contained detectable amounts of C5a or $C5a_{desarg}$, indicating that these compounds were not present at concentrations detectable in this assay (~ 8.0×10^{-12} M). As C5a is chemotactically active

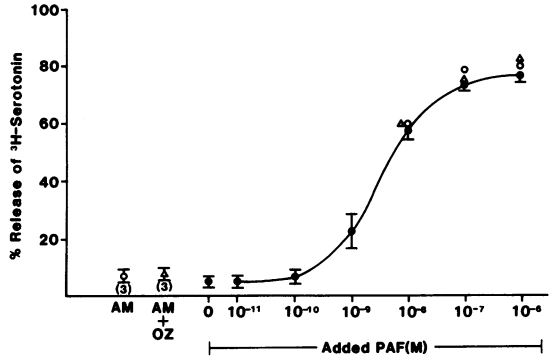


Figure 5. Assay of stimulated AM supernatants for PAF activity (mean±SD). Rabbit platelets were labeled with [^{3}H]serotonin, then incubated with aliquots of zymosan-stimulated AM supernatants or with increasing concentrations of authentic PAF to generate a reference dose-response curve. The numbers in parentheses indicate the number of separate experiments using AM from different volunteers. Also shown are the effects of adding known concentrations of authentic PAF (10^{-8}–10^{-6} M) to the three separate zymosan-stimulated (△) and -unstimulated (○) macrophage supernatants. In each case, the full activity of the added PAF was detectable. (●) Reference values.

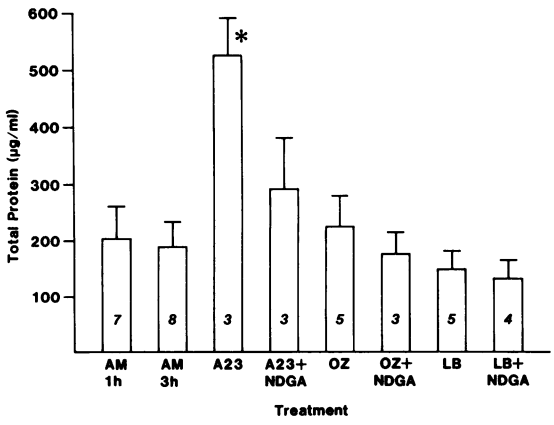


Figure 6. The effects of NDGA on protein secretion by human AM (mean±SE). The AM were stimulated with A23187, OZ or LB, as described, and the protein content in the supernatants was measured by the Lowry method. The numbers in the bars indicate the number of separate experiments, each using AM from a separate volunteer. *$P < 0.025$ compared with AM incubated alone for 1 h.

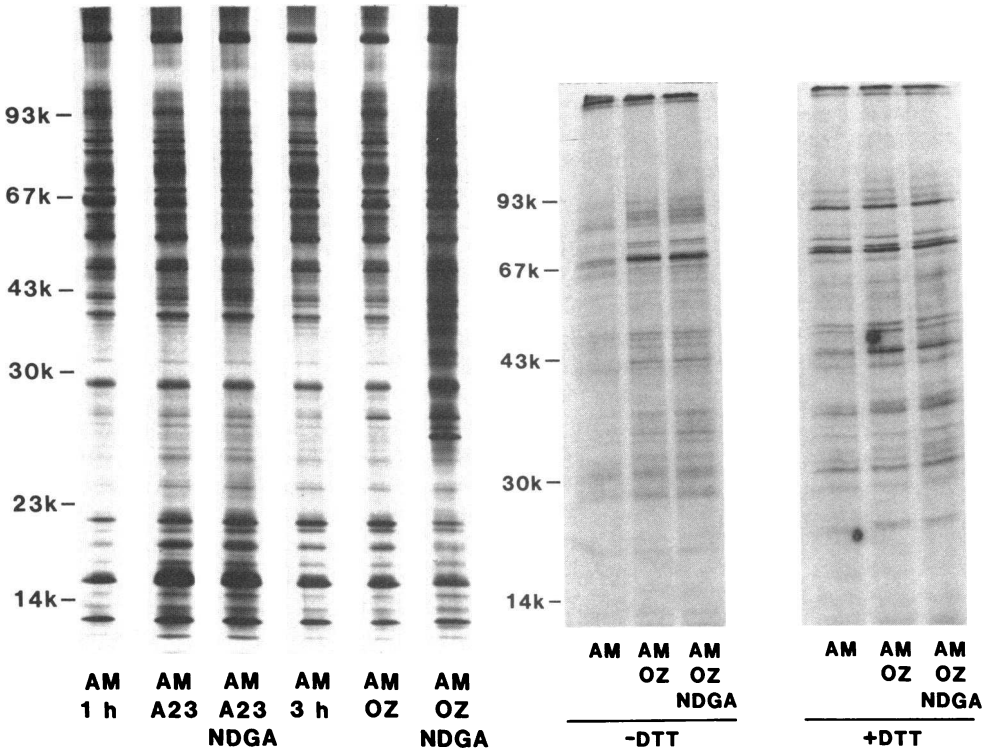


Figure 7. (*Left*) SDS-PAGE analysis of proteins in AM supernatants. Equivalent volumes of each supernatant were loaded onto a 5–15% gradient gel, which was stained with a silver stain. The macrophages were incubated as indicated either alone, or with A23187 (A23, 10 μg/ml) or OZ (1 mg/ml) with or without NDGA. (*Right*) Autofluorograph of [^{35}S]methionine-labeled proteins secreted by AM stimulated with OZ (milligrams per milliliter) in the presence or absence of NDGA (10^{-4} M). Dithiothreitol (DTT) was used as a reducing agent. Equivalent amounts of protein (5 μg) were loaded in each lane.

at concentrations > 10^{-10} M, C5 fragments could not account for the chemotactic activity in the unconcentrated macrophage supernatants.

Studies with cultured macrophages. In additional experiments, AM were incubated overnight in complete tissue culture media with or without OZ and NDGA to determine the effects of lipoxygenase blockade on neutrophil chemoactic activity liberated under these circumstances. The AM incubated alone for 24 h released detectable but small amounts of neutrophil chemotactic activity into the supernatants (Fig. 8). As with the 3-h experiments, NDGA blocked the increase in neutrophil chemotactic activity from AM incubated with OZ. The NDGA (at 10^{-4} M) did not block the basal secretion of small amounts of neutrophil chemotactic activity by the macrophages. The amount of chemotactic activity in these unconcentrated supernatants was less than in the short-term studies (Fig. 1), and chemotactic activity could not be eluted consistently from the Sephadex G-50 column. In two separate experiments with AM from different volunteers, LTB_4 was detectable by radioimmunoassay in zymosan-stimulated supernatants that had measurable neutrophil chemotactic activity (0.83±0.13 ng/10^6 cells), but it was not detectable in the corresponding NDGA-blocked supernatants that had little chemotactic activity.

Table II. Effect of Concentration on the Neutrophil Chemotactic Activity in AM Supernatants

Sample	AM + A23187	AM + A23187 + NDGA
	PMN/10 HPF	*PMN/10 HPF*
Original sample	144.3	4.0
Concentrate	91.5	1.5
Filtrate	157.8	2.0

The supernatants of AM from two different volunteers that were incubated with A23187 (10 μg/ml) with or without NDGA (1×10^{-4} M) were combined and concentrated approximately fivefold by positive pressure filtration as described in Methods. The neutrophil chemotactic activity was measured in the two original unconcentrated supernatants, the concentrate, and the filtrate, as described in Methods. The data represent the mean values of two separate experiments with each sample.

Discussion

The goal of this study was to measure the contribution of LTB_4 to the total neutrophil chemotactic activity released from the normal resident human AM. The results suggest that LTB_4 accounts for essentially all of the chemotactic activity in unconcentrated supernatants of normal human AM stimulated with the calcium ionophore A23187, and three different opsonized particulates used as phagocytic stimuli, OZ, LB, and *S. aureus*. The calcium ionophore causes rapid intracellular calcium fluxes, activating calcium-dependent pathways, including membrane phospholipases that cleave arachidonic acid from membrane fatty acids (30). The opsonized particulate stimuli are tests of Fc and complement receptor–mediated phagocytosis, although some evidence suggests that unopsonized zymosan can be phagocytized via mannose receptors on AM (31). Blockade of arachidonic acid metabolism by NDGA, a relatively specific lipoxygenase inhibitor, blocked up to 100% of the release of neutrophil chemotactic activity from AM stimulated with A23187, and each of the different particulate stimuli. The NDGA also blocked the low level of spontaneous release of chemotactic activity from AM incubated alone for 1 or 3 h. In addition, when AM were incubated overnight with OZ, NDGA also blocked the accumulation of neutrophil che-

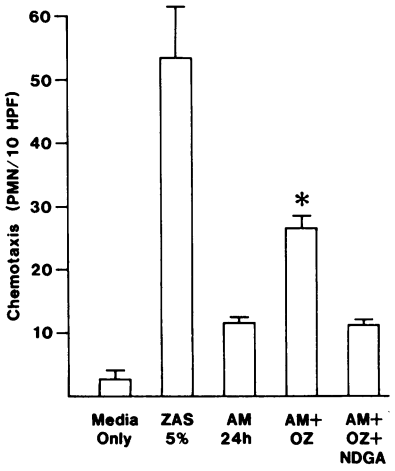


Figure 8. The effect of NDGA on neutrophil chemotactic activity in the supernatants of AM incubated for 24 h in RPMI 1640 in the presence or absence of OZ and NDGA (10^{-4} M). The untreated macrophages released low levels of neutrophil chemotactic activity. The OZ significantly increased the neutrophil chemotactic activity in the supernatants, and this effect was blocked by NDGA ($*P < 0.01$). Shown for comparison is the neutrophil chemotactic activity in zymosan-activated human serum (5% dilution in phosphate-buffered saline).

motactic activity in the macrophage supernatants. The NDGA did not affect the viability of the macrophages, or the number of cells that ingested particles, so that the results cannot be explained either by differences in cell viability or phagocytosis. These findings demonstrate that blockade of the lipoxygenase pathway inhibits the release of neutrophil chemotactic activity and suggest that the chemotactic activity released under these conditions is either a lipoxygenase product, of which LTB_4 has the most potent chemotactic activity, or that its release requires an intact lipoxygenase pathway.

The reduction in chemotactic activity caused by lipoxygenase inhibition was accompanied by a near total inhibition of LTB_4 release in the supernatants, as measured quantitatively by HPLC. Experiments with macrophages labeled with [^{3}H]arachidonic acid confirmed that NDGA blocked the release of newly synthesized LTB_4 and HETEs from the macrophages after A23187 and particulate stimuli. Furthermore, the chemotactic activity in the blocked supernatants could be reconstituted by the addition of LTB_4 in concentrations similar to those present in the unblocked supernatants.

Proof that alteration of LTB_4 release caused the change in chemotactic activity in the macrophage supernatants required exclusion of the possibility that NDGA might block the secretion of chemotactically active proteins or other lipids by the AM. The calcium ionophore A23187 is known to cause the release of granular enzymes from neutrophils (32–34), and this can be prevented by 5,8,11,14-eicosatetraynoic acid (ETYA), a combined cyclooxygenase and lipoxygenase inhibitor (33, 34). We found that NDGA did cause some reductions in the protein concentrations in the supernatants, but the profile of proteins released was not changed. When molecular sieve chromatography was used to estimate the apparent molecular weight of the chemotactic substance, we found that all of the chemotactic activity from both A23187 and zymosan-stimulated macrophages co-eluted from a Sephadex G-50 column with authentic [^{3}H]LTB_4 and not with the protein-containing fractions. These experiments place the apparent molecular weight of the chemotactic activity at < 1,000, corroborating findings with human (3) and guinea pig AM (5). This size is consistent with LTB_4 (336 mol wt) and is smaller than the smallest plasma-derived chemotactic protein, C5a (12,500 mol wt). Furthermore, the chemotactically active complement products C5a and $C5a_{desarg}$ were not detected by radioimmunoassay in the macrophage supernatants. These findings are consistent with reports indicating that antibodies to C5a did not block the neutrophil chemotactic activity in AM supernatants (2, 3), and with recent studies by Pennington and associates, who failed to find evidence of C5 by immunodiffusion in the supernatants of human AM (35).

Several additional lines of evidence suggest that proteins do not contribute significantly to the neutrophil chemotactic activity in the unconcentrated AM supernatants. No chemotactic activity was detected in the protein-containing fractions from the Sephadex G-50 column, making it unlikely that proteins in the unconcentrated supernatants contribute directly to the chemotactic activity, or indirectly by binding significant amounts of lower molecular weight species. Analysis of the macrophage supernatants by SDS-PAGE showed that NDGA did not change the protein profiles in the supernatants. The experiments with [^{35}S]methionine-labeled macrophages showed that NDGA also did not alter the pattern of newly synthesized proteins released in response to these stimuli. When the stimulated AM supernatants were concentrated, the chemotactic activity was present in the concentrate as well as the filtrate, suggesting that the active species distributes on both sides of a 5,000-mol-wt limit membrane, as would be the case for a low molecular weight species. Analysis of the concentrate alone could have suggested incorrectly that the active species was larger than 5,000 mol wt. Furthermore, concentration of an NDGA-blocked, chemotactically inactive supernatant failed to augment chemotactic activity, as would be expected if there were low concentrations of a chemotactically active protein that was unaffected by NDGA. Therefore, the data from molecular sieve chemotography, SDS-PAGE, concentration of the supernatants, and the C5a radioimmunoassay all suggest that proteins do not contribute significantly to the neutrophil chemotactic activity released in 1–3 h by the normal human AM in response to the calcium ionophore and the particulate stimuli that we studied.

The additional experiments that we performed using AM incubated overnight indicate that under these conditions also, LTB_4 is likely to be the predominant neutrophil chemoattractant released. The NDGA blocked the increase in chemotactic activity caused by phagocytosis of OZ and this correlated with blockade of LTB_4 release. The persistent background chemotactic activity in these supernatants, however, leaves open the possibility that these supernatants also contain small amounts of another chemoattractant, e.g., the larger molecular weight species described by Merrill and associates in the 10-fold concentrated supernatants of normal AM incubated for 24 h in tissue culture media (2).

PAF is another low molecular weight lipid product that is produced by both mononuclear and polymorphonuclear phagocytes and is chemotactically active for neutrophils and monocytes, as is LTB_4 (17, 18, 36–39). In rat AM, PAF is formed by the actions of phospholipase A_2 and acetyl transferase on membrane phospholipids in the same sequence of reactions that leads to the release of arachidonic acid (40, 41). The observation that the supernatants of ionophore-stimulated human AM cause platelet aggregation (42) suggested that these cells also produce PAF, and raised the possibility that PAF

release might account for some of the chemotactic activity in these stimulated macrophage supernatants.

Our results suggest that PAF does not account for the neutrophil chemotactic activity that we observed for several reasons. First, the molecular sieve chromatography data indicate that the chemotactic activity produced by ionophore- and zymosan-stimulated macrophages eluted later than PAF. Authentic PAF consistently eluted at least four tubes earlier than both authentic LTB_4 and the peak of the chemotactic activity. When authentic ^{3}H-PAF and [^{3}H]LTB_4 were mixed and added to the Sephadex G-50 column, twin peaks were recovered at the locations corresponding to those observed when each was added separately. Second, PAF was not recovered by HPLC from the supernatants of ionophore-stimulated AM. Third, supernatants of zymosan-stimulated macrophages that contained neutrophil chemotactic activity did not cause the release of [^{3}H]serotonin from rabbit platelets, which is a sensitive bioassay for PAF activity (18). When authentic PAF was added to these supernatants, the expected amount of [^{3}H]-serotonin release occurred, indicating that the supernatants did not contain a macrophage product that blocked PAF-induced serotonin release. Fourth, we were unable to reconstitute the chemotactic activity in the NDGA-blocked supernatants by the addition of authentic PAF (1.0×10^{-7} M).

In summary, the data indicate that LTB_4 is the predominant neutrophil chemoattractant produced by the normal human AM in response to calcium flux across the cell membrane and the phagocytosis of opsonized particulates. Our findings are limited to normal AM, as we obtained macrophages from humans without chronic inflammatory lung diseases and we did not study the effects of immunologic activation. It is possible that when AM are activated, the profile of chemoattractants produced changes and that under these conditions, protein or peptide chemoattractant(s) might be more important (2). Further studies are warranted with activated AM derived from patients with chronic immunologic lung diseases and with AM activated in vitro with different immunologic stimuli, e.g., γ-interferon or other lymphokines (43).

The finding that normal resident AM secrete large amounts of LTB_4 suggests that these cells may play an important role in the initiation and the amplification of inflammatory processes in response to inhaled stimuli, by recruiting neutrophils and monocyte–macrophages into the lung. The finding that LTB_4 also is a chemoattractant for fibroblasts suggests a role for this AM-derived product in fibrotic responses as well (44). As LTB_4 appears to be the major neutrophil chemoattractant elaborated by normal resident human AM, pharmacologic modulation of its release might help to limit some types of inflammatory reactions in the lungs.

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