Macrophages Cultured In Vitro Release Leukotriene B_4 and Neutrophil Attractant/Activation Protein (Interleukin 8) Sequentially in Response to Stimulation with Lipopolysaccharide and Zymosan

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Abstract

The capacity of lipopolysaccharide (LPS), zymosan, and calcium ionophore A23187 to induce neutrophil chemotactic activity (NCA), leukotriene B_4 (LTB_4), and neutrophil attractant/activation protein (NAP-1) release from human alveolar macrophages (AM) retrieved from normal nonsmokers was evaluated. LPS induced a dose-dependent release of LTB_4 that began by 1 h, 4.0±3.2 ng/10^6 viable AM; peaked at 3 h, 24.7±13.5 ng/10^6 viable AM; and decreased by 24 h, 1.2±1.0 ng/10^6 viable AM (n = 8). Quantities of LTB_4 in cell-free supernatants of AM stimulated with LPS were determined by reverse-phase high-performance liquid chromatography and corresponded well with results obtained by radioimmunoassay. By contrast, NAP-1 release began ~ 3–5 h after stimulation of AM with LPS, 197±192 ng/ml, and peaked at 24 h, 790±124 ng/ml. Release of NAP-1 was stimulus specific because A23187 evoked the release of LTB_4 but not NAP-1, whereas LPS and zymosan induced the release of both LTB_4 and NAP-1. The appearance of neutrophil chemotactic activity in supernatants of AM challenged with LPS for 3 h could be explained completely by the quantities of LTB_4 present. After stimulation with LPS or zymosan for 24 h, AM had metabolized almost all generated LTB_4. Preincubation of AM with nordihydroguiaretic acid (10^{-4} M) completely abolished the appearance of NCA, LTB_4, and NAP-1 in supernatants of AM challenged with LPS. Therefore, LPS and zymosan particles were potent stimuli of the sequential release of LTB_4 and NAP-1 from AM. (*J. Clin. Invest.* 1990. 86:1556–1564.) Key words: alveolar macrophages • leukotriene B_4 • neutrophil attractant/activation protein • interleukin 8 • lipopolysaccharide • zymosan

Introduction

Alveolar macrophages (AM)[1] reside on the epithelial surfaces of human large airways (1, 2) and alveoli (3). These cells serve several functions which include important roles in host lung defense (4, 5) and in inflammatory processes central to several lung diseases (3, 6). Macrophages release an impressive array of biologically active molecules (7) among which are molecules with the ability to direct the migration of neutrophils to the lung.

Leukotriene B_4 (5S,12R)-dihydroxy-(6Z,8E,10E,14Z)-eicosatetraeonic acid (LTB_4) is one human AM product (8–13) with numerous effects on a variety of leukocytes. Of particular interest are the effects of LTB_4 on human neutrophils. These include potent chemokinetic and chemotactic activity (14–17), induction of aggregation (15), and stimulation of neutrophil lysosomal enzyme release (18). Another human AM product with a profound ability to modulate neutrophil function is a recently isolated (19, 20), sequenced (21–23), and cloned (24) 72-residue neutrophil attractant/activation protein (NAP-1), which is also referred to as NAP-1/IL-8.[2] Both NAP-1 that is generated from lipopolysaccharide (LPS)-stimulated human peripheral blood monocytes (19) and recombinant NAP-1 are potent chemotaxins for human neutrophils (25).

Whereas human AM release significant quantities of LTB_4 within minutes after stimulation with A23187 (8, 10–13, 26) or zymosan (26) and large amounts of NAP-1 by 24 h after stimulation with LPS (27), we hypothesized that A23187, zymosan, and LPS would elicit the release of LTB_4 first and at later time points NAP-1, and that each of these neutrophil chemotaxins might contribute, at junctures consistent with their time course of release, to the overall neutrophil chemotactic activity present in supernatants of purposefully stimulated AM. Our results demonstrate that AM release of these two neutrophil chemotaxins is stimulus specific, that AM release of LTB_4 and NAP-1 occurs in sequence, and that AM effectively metabolize LTB_4 over 24 h to at least five products separable by reverse-phase high-performance liquid chromatography (RP-HPLC). The combined ability of the macrophage to release sequentially LTB_4 and NAP-1, and the ability of this cell to regulate extracellular concentrations of LTB_4, provide this effector cell with a graded flexibility to effect neutrophil movement to the lung.

Methods

Preparation of alveolar macrophage cultures. Normal nonsmoking volunteers were recruited from the Yale University undergraduate campus and the Yale New Haven and West Haven Veterans Hospitals by one of us (Dr. Rankin). AM were retrieved by bronchoalveolar

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1. *Abbreviations used in this paper:* AM, alveolar macrophage(s); BAL, bronchoalveolar lavage; NAP, neutrophil attractant/activation protein; RP, reverse phase (HPLC).

2. This nomenclature was presented at a 1-day international symposium entitled "Novel Neutrophil Stimulating Peptides: Source, Structure, and Role in Inflammation," London, England, 9 December 1988.

lavage (BAL) as described in detail (11). Briefly, no subject admitted to any acute or chronic illness, none was taking any regular medication other than birth control pills, and none admitted to an acute respiratory tract illness in the 4-wk period before participation. All subjects had normal spirometry and chest roentgenograms. Topical 4% lidocaine was used to anesthetize the oropharynx. BAL then was performed through a fiberoptic bronchoscope, wedged in a segmental bronchus, by instilling pyrogen-free sterile normal saline in 50-ml aliquots and gently aspirating after each instillate. A maximum of 250 ml was instilled into each lung. BAL aliquots then were pooled, filtered through a single layer of gauze, and centrifuged at 500 *g* for 5–10 min at 24°C. The cells were washed one to two times in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS, Grand Island Biological Co., Grand Island, NY) and resuspended in LPS-free RPMI-1640 containing 1% fetal bovine serum, 2 mM L-glutamine, and 50 μg/ml of gentamicin sulfate at 2×10^6 cells per ml. Cell viability was determined by the exclusion of Trypan blue. The total number of cells was enumerated on a hemacytometer, and differential cell counts were performed on cytocentrifuge preparations stained with Diff-Quik (American Scientific Products, McGaw Park, IL). Lavage cells prepared in this fashion totaled (mean±SD) 85.1±19.0% AM, 9.3±4.7% lymphocytes, 0.7±0.7% neutrophils, and 0.3±0.4% eosinophils (n = 23). Macrophage viability prior to adherence averaged 88.2±4.6%; red blood cell contamination averaged 8.0±3.7%. Platelet contamination, estimated from the small number of red blood cells in the lavage fluids never exceeded one platelet per nucleated cell. One million cells then were added to sterile 24-well culture plates (Costar, Cambridge, MA) and adhered for 1 h before the nonadherent cells were removed gently with a single wash of medium. The cells then were covered with 0.5 ml of medium.

Preparation of AM stimuli. A23187 was purchased from Calbiochem-Behring Corp., La Jolla, CA, dissolved in DMSO, diluted to 200 μM in HBSS, and stored at −70°C. Nordihydroguiaretic acid (NDGA), purchased from Sigma Chemical Co., St. Louis, MO, was resuspended in ethanol at 10^{-2} M and stored at −70°C.

Zymosan (Sigma Chemical Co.) particles were prepared by suspending them in endotoxin-free normal saline and boiling for 1 h. The particles were centrifuged at 100 *g* for 10 min to remove any aggregates. The remaining particles were counted in a counter (Coulter Electronics, Inc., Hialeah, FL) and adjusted to a concentration that resulted in a ratio of ~ 50 particles per macrophage in the AM cultures. Soluble β-D-glucan (Laminarin, Sigma Chemical Co.) was resuspended at 5 mg/ml in culture medium and stored at −70°C.

LPS, *Escherichia coli* LPS 055:B5, purchased from Difco Laboratories, Inc., Detroit, MI, was resuspended in the RPMI-1640 medium used for the cell cultures at a concentration of 1 mg/ml.

Quantitation of NAP-1. NAP-1 was quantified using a sandwich enzyme-linked immunosorbent assay (ELISA). The production, cloning, assay, and purification of mouse monoclonal IgG anti-NAP (28) and other details of the assay have been described previously (27). Mouse monoclonal anti-NAP-1 was used as capture antibody at a concentration of 9 μg/ml. Nonspecific binding was prevented by 0.1% bovine serum albumin. Polyclonal rabbit anti-NAP-1 was used as detection antibody. After incubation with alkaline phosphatase–conjugated goat anti-rabbit IgG (Sigma Chemical Co.), we used the ELISA Amplification System (Bethesda Research Laboratories, Gaithersburg, MD) to increase sensitivity. Serial NAP-1 dilutions were applied to each plate as a reference standard. Absorbance was recorded at 490 nm with an automatic ELISA reader. This assay is sensitive to NAP-1 concentrations of ~ 1 ng/ml. Two human platelet proteins with sequence similarity to NAP-1, platelet factor 4, and connective tissue-activating protein III (CTAP-III), did not cross-react in the sandwich ELISA for NAP-1. In addition, the ELISA for NAP-1 did not demonstrate any cross-reactivity with standard NAP-2 over a concentration range of 1–3,125 ng/ml.

Neutrophil chemotaxis assay. Neutrophil chemotaxis was assayed in multiwell chemotaxis chambers. A 10-μm-thick polyvinylpyrrolidone-free 3-μm pore size polycarbonate filter separated the well with neutrophils (5×10^4 cells per well) from the well with attractant (27). The chambers were incubated for 40 min at 37°C in humidified air-5%CO_2 and the filters were removed. Nonmigrated neutrophils were wiped away and the filters were air-dried and stained with Diff-Quik. Neutrophils that migrated through the pores to the attractant side of the membrane were counted with an image analyzer. Attractants were assayed in duplicate and migrated cells in three randomly selected fields of each well, representing ~ 20% of the assay well surface, were counted. Percent migration was calculated from the total number of cells applied per well (5×10^4), well area (8 mm), field area counted (0.5 mm^3 per field), and the actual number of cells per field seen. FMLP at 10^{-7} was used as a reference chemoattractant; HBSS was used as the negative control. A dose-response curve to LTB_4 was determined using synthetic standard provided by Dr. J. Rokach, Merck Frosst, Point Le-Claire, Quebec, Canada. LTB_4 was stored in methanol and maintained under nitrogen, and dilutions were made in HBSS. HBSS with an equivalent percentage of methanol served as the negative control. Data are presented as the percent migration of input neutrophils±SD for duplicate wells (27).

Assessment of the metabolism of LTB_4 by cultures of AM. The ability of cultures of AM to metabolize LTB_4 was determined by incubating AM monolayers at 1×10^6 AM/ml for 24 h at 37°C in the presence of 20,000–30,000 cpm/10^6 cells of [^{3}H]LTB_4 and with or without LPS (10 μg/ml). In one additional experiment, AM monolayers were incubated with [^{3}H]LTB_4 and standard LTB_4 (50 ng/ml) for 24 h at 37°C. AM supernatants were removed and centrifuged at 500 *g* for 5–10 min at 4°C. 50-μl aliquots of cell-free supernatants were diluted in 10 ml of Ecoscint (National Diagnostics, Inc., Manville, NJ) and assessed for radioactivity. The remainder of the supernatant was added to 2 ml of 100% methanol, stored at 4°C for at least 30 min, and deproteinated by centrifugation at 1,000 *g* for 30 min at 4°C. The supernatant fluid was evaporated to dryness under negative pressure, and the sediment was resuspended in mobile phase and resolved by RP-HPLC. An aliquot of the pre-RP-HPLC suspension and each column fraction also were assessed for radioactivity. Control cultures consisted of medium with [^{3}H]LTB_4 and LPS incubated in the absence of AM for the same time period and processed identically.

Quantitation of LTB_4 by Radioimmunoassay (RIA) and RP-HPLC. LTB_4 was quantified with an RIA described previously (11), using rabbit immune plasma provided by Dr. A. Ford-Hutchinson, Merck Frosst, and [^{3}H]LTB_4 (184 Ci/mM, New England Nuclear, Boston, MA). This assay employs Dextran-coated charcoal to separate antibody-bound LTB_4 from unbound LTB_4 and is sensitive to 0.3 ng/ml. Quantities of LTB_4 are expressed in nanograms per 10^6 viable macrophages. Corresponding values in nanograms per milliliter of AM supernatant are provided where indicated.

RP-HPLC was performed using a Waters Associates (Milford, MA) system equipped with a Nova Pak C18 column (3.9 × 150 mm). Samples were eluted isocratically with a mobile phase consisting of methanol/water/acetic acid (67:33:0.08, vol/vol/vol; pH adjusted to 5.7 with ammonium hydroxide) at a flow rate of 1 ml/min with continuous online monitoring at 280 nm. Synthetic LTB_4, 20-OH-LTB_4, and 20-COOH-LTB_4 standards were provided by Dr. J. Rokach, Merck Frosst. [^{3}H]LTB_4 standard (32.8 Ci/mM) was purchased from New England Nuclear. Fractions for determination of radioactivity were collected using a Frac-100 (Bio-Rad Laboratories, Richmond, CA).

Statistical analyses. All data are expressed as the mean±SD. Data analyses were performed using the Wilcoxon signed rank test for matched pairs or with a paired t test using the CRUNCH statistical software package (CRUNCH Software Corp., Oakland, CA). P values ≤ 0.05 were considered significant.

Results

LTB_4 release from human AM stimulated with LPS. In previous studies (27), we determined that adherent human AM

incubated for 24 h in vitro with LPS released substantial quantities of NAP-1. Thus, we examined whether or not the incubation of human AM with LPS also would induce the release of LTB_4. The time-dependent release of LTB_4 from LPS-stimulated AM is shown in Fig. 1. Duplicate samples for each time point were assessed by both RIA and RP-HPLC in each of three experiments. LTB_4 present in supernatants of AM cultured with LPS for 0.1 and 0.5 h was < 0.6±0.1 ng/10^6 viable AM for each time point, as determined by RIA. After 1 h LPS induced the release of 3.5±3.9 ng of immunoreactive LTB_4/10^6 viable AM, which corresponds to 5.1±4.2 ng/ml. Product generation peaked at 3 h, 19.6±10.2 ng/10^6 viable AM (24.5±9.7 ng/ml), and amounts present at 24 h had declined to 0.9±0.5 ng/10^6 viable AM (1.5±0.8 ng/ml). Determination of the amounts of LTB_4 by RP-HPLC in aliquots of the same supernatants revealed a similar time-response curve. At the 1-h time point, the material eluting at the retention time of standard LTB_4 contained 3.3±3.8 ng/10^6 viable AM by integrated optical density, which represents 94.3% of that determined by RIA. At the 3-h time point, the material eluting at the retention time of synthetic LTB_4 contained 13.1±7.6 ng/10^6 viable AM, which is 66.8% of that detected by RIA. At the 5-h time point, 6.6±6.2 ng/10^6 viable AM were found by RP-HPLC, which was 46.5% of that determined by RIA. By 24 h after stimulation with LPS, < 1.3 ng of LTB_4 was detected by either RIA or RP-HPLC. Thus, LPS was an effective stimulus for AM release of LTB_4 quantitated by both RIA and RP-HPLC.

The dose dependency of LPS-induced LTB_4 release was determined using concentrations ranging from 2 ng to 20 μg/ml on AM incubated for 3 h at 37°C (Table I). The LPS dose response of LTB_4 release, as determined by RIA, was progressive up to a concentration of 2 μg/ml and plateaued.

LPS- and zymosan-induced NCA, LTB_4, and NAP-1 release. Duplicate cultures of AM were challenged with either LPS (10 μg/ml) or zymosan (particle/AM ratio of 50:1) for time periods from 0.1 to 24 h. Neutrophil chemotactic activity (NCA), LTB_4, and NAP-1 were determined on aliquots of duplicate samples. Neutrophil chemotactic activity increased rapidly in supernatants from AM stimulated with each substance from 0.1 to 3 h and then more slowly to the last time point tested, 24 h (Fig. 2, *A* and *B*). For AM stimulated with LPS, LTB_4 was detected by 1 h, 4.3±3.2 ng/10^6 ng viable AM (7.8±5.9 ng/ml); peaked at 3 h, 27.8±15.3 ng/10^6 viable AM (46.4±22.7 ng/ml); and declined by 24 h to 1.2±1.3 ng/10^6 viable AM (2.0±1.9 ng/ml). When the results from all supernatants of AM challenged with LPS (Figs. 1 and 2 *A*) were combined (n = 8), cells released 4.0±3.2 ng/10^6 viable AM (6.7±5.5 ng/ml) by 1 h and 24.7±13.5 ng/10^6 viable AM (41.0±21 ng/ml) by 3 h. LTB_4 concentrations in supernatants were minimal, 1.1±1.0 ng/10^6 viable AM (1.8±1.7 ng/ml), at 24 h. Zymosan-induced LTB_4 release began slightly earlier at 30 min, 2.4±0.8 ng/10^6 viable AM (3.6±1.0 ng/ml) and peaked at 3 h, 16.2±5.0 ng/10^6 viable AM (25.6±6.4 ng/ml). By 24 h only minimal quantities of LTB_4 were detectable, 0.5±0.1 ng/10^6 viable AM (0.9±0.1 ng/ml).

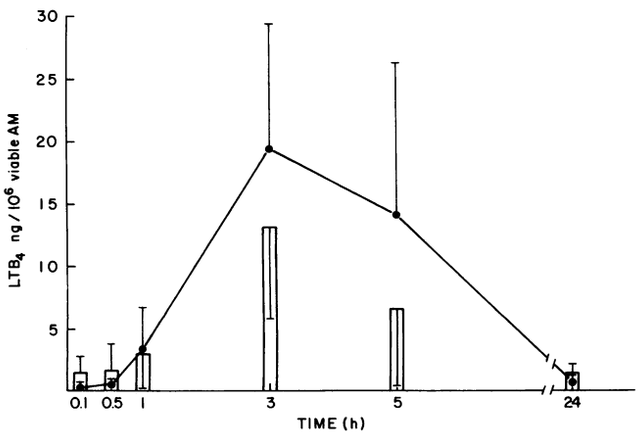

Figure 1. LPS-induced LTB_4 release from human AM. AM were incubated for the indicated times with LPS (10 μg/ml). Quantitation of LTB_4 was determined by RIA (*solid line*) and RP-HPLC (*bars*) for duplicate samples in each experiment. Data are the mean±SD of three experiments.

Table I. Dose-dependent Effect of LPS on Alveolar Macrophage Release of LTB_4

AM donor	LPS 0	0.002	0.02	0.2	2	20
			μg/ml			
			ng/10^6 viable AM			
1	0.3	10.1	27.4	28.7	30.5	25.0
2	0	4.3	7.7	9.9	14.2	12.0

Data are from supernatants of AM cultured with the indicated concentrations of LPS for 3 h at 37°C and assessed by RIA.

The release of NAP-1 in response to LPS demonstrated a distinctly different time course than that of LTB_4. NAP-1 was first detected 5 h after stimulation, 197±192 ng/ml, and was highest at the 24 h (Fig. 2 *A*). The mean 24-h value for three experiments was 790±124 ng/ml (~ 1×10^{-7} M) for LPS-stimulated AM and 260±180 ng/ml from paired cultures of AM that were not purposefully activated, $P < 0.05$. In each of the three paired experiments, quantities of NAP-1 present in supernatants of zymosan-stimulated AM, 270±70 ng/ml, were greater than those present in the supernatants of not purposefully activated AM, 150±140 ng/ml, but this did not reach statistical significance. When these data were pooled with results from an additional two experiments, concentrations of NAP-1 in 24-h supernatants of zymosan-stimulated AM, 530±410 ng/ml, were significantly greater than those in supernatants of not purposefully activated AM, 310±240 ng/ml, $P = 0.04$. While both stimuli induced the release of NAP-1 by 24 h that was significantly greater than that observed from not purposefully activated AM, quantities of NAP-1 present in the supernatants of not purposefully activated AM were significantly greater at 24 h, 270±70 ng/ml, than at 3 h, 4±7 ng/ml, $P = 0.03$, suggesting that secretion of NAP-1 by these AM could reflect their condition in vivo or could be a result of stimulation associated with collection or culture. The 24-h ELISA NAP-1 concentrations in supernatants of both LPS- and zymosan-stimulated AM at 24 h are on the plateau of the NAP-1 neutrophil chemotaxis curve.

To assess the specificity of zymosan-induced LTB_4 release, we tested the ability of soluble β-D-glucans to inhibit particulate zymosan-induced and LPS-induced LTB_4 release from AM. Laminarin was preincubated with the AM for 40 min

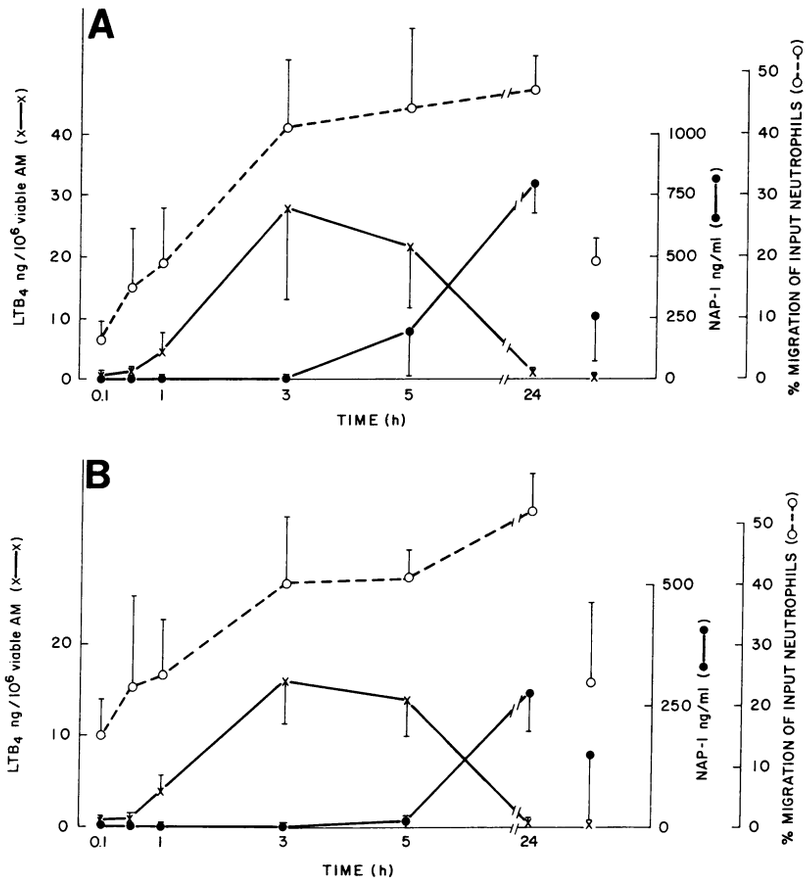

Figure 2. Neutrophil chemotaxis, LTB_4, and NAP-1 determinations on supernatants from (*A*) LPS-stimulated (n = 5) and (*B*) zymosan-stimulated (n = 3) AM. Neutrophil chemotaxis (○), LTB_4 (×), and NAP-1 (●) were assessed on duplicate supernatants from each experiment at the indicated time points. Results on supernatants from AM cultured for 24 h in the absence of agonist are shown by the solitary symbols. Data are the mean±SD.

before the addition of zymosan, and the incubation was continued for an additional 3 h in the continued presence of laminarin. Laminarin inhibited LTB_4 release in response to zymosan by a mean of 39.2±22.4% (Table II). In contrast, LTB_4 release induced by LPS, 13.5±9.2 ng/10^6 viable AM, was not inhibited by laminarin, 15.5±7.4 ng/10^6 viable AM (n = 3).

Determination if chemotactic activity in 3-h LPS-stimulated AM culture fluids was attributable completely to LTB_4. AM were stimulated for 3 h at 37°C with 10 μg/ml of LPS (n = 3), and LTB_4 concentrations in these supernatants were determined by RIA. The neutrophil chemotactic activity of synthetic LTB_4 and that of three serial dilutions of an aliquot of supernatants from LPS-stimulated AM then was determined using neutrophils from a single donor. The neutrophil chemotactic responses of these supernatants closely approximated the neutrophil chemotactic activity induced by equivalent concentrations of synthetic LTB_4 (Fig. 3). These data suggest

Table II. Laminarin Inhibition of Zymosan-stimulated LTB_4 Release from Human Alveolar Macrophages

AM donor	Zymosan	Zymosan + laminarin	Laminarin	Inhibition
		ng/10^6 viable AM		%
1	12.2	5.3	2.1	68.3
2	16.3	12.4	6.9	41.5
3	5.7	5.0	1.0	14.8
4	6.6	5.0	1.6	32.0
Mean	10.2	6.9	2.9	39.2
±SD	±5.0	±3.7	±2.7	±22.4

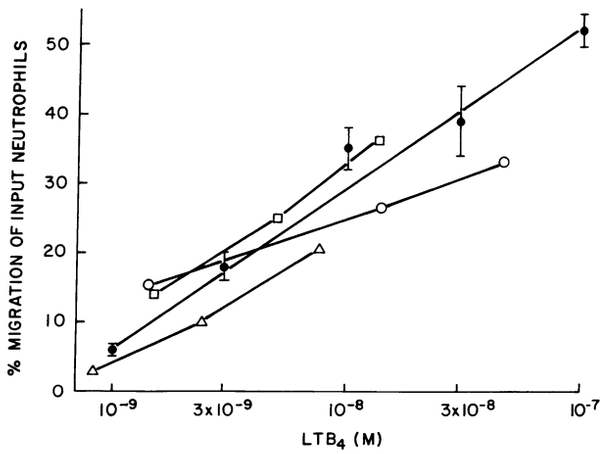

Figure 3. Neutrophil chemotactic activity of synthetic LTB_4 and of supernatants of AM stimulated for 3 h with LPS (10 μg/ml). Neutrophil chemotactic activity of AM supernatants was determined at three serial dilutions. Mean (○, □, △) results from AM of three separate subjects. The concentration of LTB_4 in each supernatant was determined by RIA. (●) Neutrophil chemotactic activity induced by synthetic LTB_4. Bars represent standard deviations.

that all of the neutrophil chemotactic activity present in supernatants of AM challenged with LPS for 3 h could be accounted for by the quantities of LTB_4 present.

A23187-induced release of NCA, LTB_4, and NAP-1. Duplicate cell cultures were incubated with A23187 for time points from 0.1 to 24 h, and aliquots of the AM supernatants were assessed for NCA, LTB_4, and NAP-1. AM release of NCA, LTB_4, and NAP-1 in response to A23187 contrasted markedly with that observed from AM stimulated with either LPS or zymosan (Fig. 4). The NCA present in supernatants from AM stimulated with A23187 was greater at the 0.1-h time point than that in the 0.1-h time points from cells stimulated with LPS or zymosan. This was associated with the presence of relatively large quantities LTB_4 in the supernatants of A23187-stimulated AM, 11.1±3.7 ng/10^6 viable AM (14.6±8.1 ng/ml), relative to the concentrations of LTB_4 present in the 0.1-h time points of AM stimulated with zymosan or LPS, which never exceeded 1.1±0.3 ng/10^6 viable AM. In contrast to results obtained from experiments using either LPS or zymosan as stimuli, the time-response curve for the generation of NCA induced by A23187 peaked at 0.5 h and declined slowly but progressively over the subsequent 24 h. The time-response curve for the release of LTB_4 closely paralleled the NCA response curve. However, NCA and significant quantities of LTB_4, 30.8±46.0 ng/10^6 viable AM (48.5±60.8 ng/ml), continued to be present at the 24 h time point. A23187 was not an effective stimulus for NAP-1 release at any of the time points tested.

NDGA inhibition of NCA, LTB_4, and NAP-1 release. Additional experiments were performed with NDGA (10^{-4} M) to inhibit LPS-induced LTB_4 release and to assess the effect this would have on the shape of the NCA time-response curve and NAP-1 release. In two separate experiments NDGA inhibited completely all NCA, LTB_4, and NAP-1 release from AM stimulated with LPS (Fig. 5).

Metabolism of LTB_4 by AM. As shown in Fig. 1, 2, and 4, less LTB_4 remained in supernatants of AM stimulated with either LPS, zymosan, or A23187 at the 24-h time points compared to quantities present at times of peak release. This suggested that AM in the cultures were capable of metabolizing LTB_4 to products that were not recognized by the anti-LTB_4 antisera and did not absorb at 280 nm. To assess this hypothesis, we performed five experiments in which [^{3}H]LTB_4 (20,000–30,000 cpm) was incubated in culture media for 24 h at 37°C in the absence and presence of adhered AM. In each experiment > 70% of the added radiolabeled LTB_4 was recovered in the deproteinated residue that was resuspended in mobile phase just before RP-HPLC. [^{3}H]LTB_4 incubated for 24 h at 37°C in the absence of AM resolved into three major peaks; the first eluted at 2.5±0.5 min and contained 19.8±8.2% of the recovered counts; the second eluted at 7.3±0.3 min and contained 14.3±3.8% of the recovered counts; the third eluted at the known retention time of [^{3}H]LTB_4 standard for this column (9.6±0.3 min) and contained 58.8±4.6% of the recovered counts. In contrast, [^{3}H]LTB_4 incubated in the presence of AM but without LPS for 24 h resolved into five major peaks with the following retention times; peak 1, 2.4±0.3 min; peak 2, 4.6±0.5 min; peak 3, 11.2±0.6 min; peak 4, 17.4±0.5 min; and peak 5, 19.3±0.5 min (Fig. 6). The percentage of counts in peak 1, 28.9±5.4%; peak 2, 12.8±3.1%; peak 3, 13.0±6.3%; peak 4, 3.9±3.0%; and peak 5, 10.4±2.4% totaled ~ 70% of the recovered counts. In two additional experiments [^{3}H]LTB_4 incubated in the presence of adhered AM and with LPS (10 μg/ml) for 24 h at 37°C resolved into the same five major peaks and contained a similar proportion of the recovered counts (data not shown). In addition, one additional peak was observed which eluted at 8.1±0.5 min and contained 15.8±0.6% of the recovered counts. None of these peaks corresponded to the known retention time of standard [^{3}H]LTB_4 for this column. The first peak in all the above experiments, eluted at approximately the same time as the 20-OH-LTB_4 and 20-COOH-LTB_4 standards on the column, suggesting that the products in peak 1 were probably the ω-oxidation metabolites of LTB_4.

In one additional experiment LTB_4 was added at a concentration of 50 ng/ml along with [^{3}H]LTB_4 to AM that were incubated for 24 h at 37°C without LPS. The cell-free supernatant was deproteinated and resolved by RP-HPLC. In this experiment none of the LTB_4 metabolites was detected by online monitoring at 280 nm.

Discussion

The results of this study firmly establish several new findings. First, they prove that LPS *E. coli* 055:B5 will elicit the time-dependent release of LTB_4 from normal, adhered human AM cultured in vitro. The quantities of LTB_4 determined by RP-HPLC closely approximated amounts assessed at each time

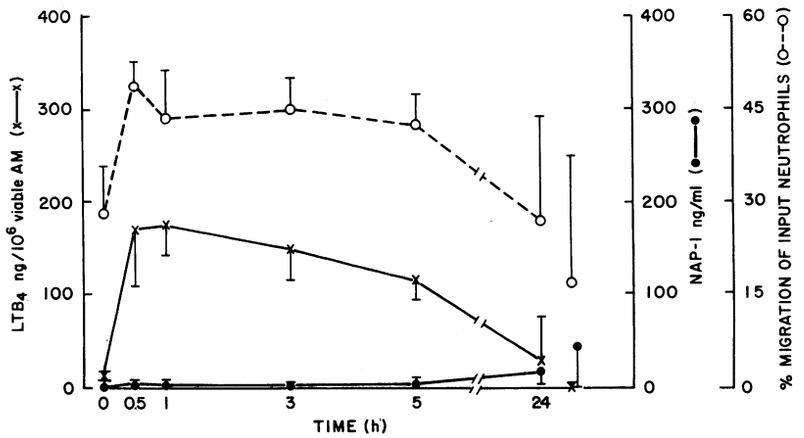

Figure 4. Neutrophil chemotaxis, LTB_4, and NAP-1 determinations on supernatants from A23187-challenged AM are depicted as in Fig. 2. Data are the mean±SD of four experiments.

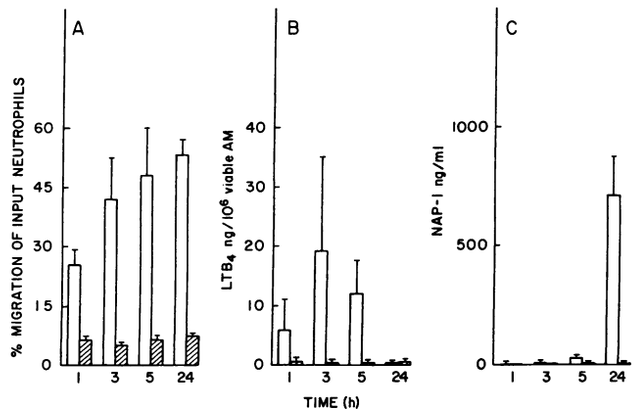

Figure 5. Effect of pretreatment of AM with NDGA. AM were incubated with NDGA (10^{-4} M) for 15 min at 37°C before stimulation with LPS (10 μg/ml) for the time points indicated. (*A*) Neutrophil chemotatic activity; (*B*) LTB_4; (*C*) NAP-1. Open bars represent results obtained in the absence of NDGA and shaded bars results obtained in the presence of NDGA. Data are the mean±SD of duplicate samples from two experiments.

point by RIA. Mean LTB_4 release, as determined by RIA, at the 3-h time point was 24.7±13.5 ng/10^6 viable AM with a range of 7.5–45 ng/10^6 viable AM which corresponds to concentrations of $\sim 2 \times 10^{-8}$ M to 1.3×10^{-7} M. These concentrations are functionally significant, considering that concentrations of 3×10^{-9} M LTB_4 are chemotactic for neutrophils (Fig. 3 and reference 29). Importantly, LPS in concentrations as low as 2 ng/ml were an effective stimulus for LTB_4 release. The amounts of LPS utilized in our studies were in the range of

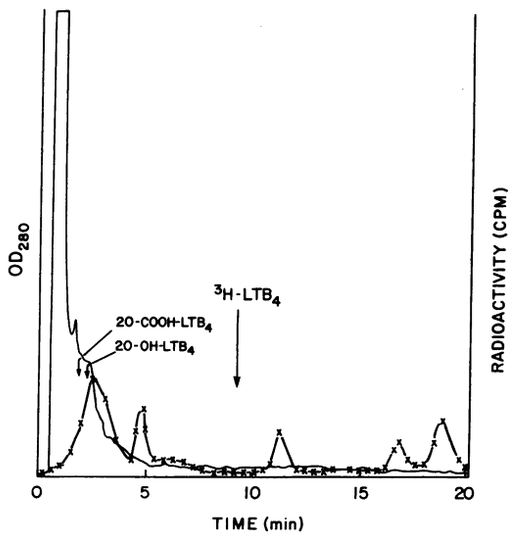

Figure 6. Effect of the incubation of [^{3}H]LTB_4 with AM (×) for 24 h at 37°C before analysis by RP-HPLC. 0.5-ml fractions were collected and assessed for radioactivity. The arrow at the retention time of 9.6 min denotes the elution times for both [^{3}H]LTB_4 standard from this column and for [^{3}H]LTB_4 present in medium cultured in the absence of AM for 24 h. The arrows at the retention time of 1.8 and 2.1 min indicate the elution times of 20-COOH- and 20-OH-LTB_4 standards, respectively. The results are the mean of duplicate determinations from a single experiment that is representative of four additional experiments.

LPS quantities detected in the plasma of patients with sepsis (30). LTB_4 has been identified in increased concentrations in the BAL fluid of both patients with the adult respiratory distress syndrome (ARDS) and those at risk for developing it (31). We have observed previously (27) and confirmed in this study that LPS also is a potent stimulus for NAP-1 release. Collectively, these observations support the hypothesis that during sepsis circulating endotoxin activates lung macrophages—possibly by binding to one or more of the LPS-binding proteins present on macrophages (32, 33)—to release LTB_4 and NAP-1, which subsequently recruit neutrophils to the lung.

The above data differ from those of Brown and colleagues (34) who determined that normal human AM release only cyclooxygenase products after challenge with endotoxin. Lüderitz and co-workers (35) have established that different LPS vary in their capacity to effect leukotriene release from mouse peritoneal macrophages. Thus, the difference between the findings of Brown and co-workers and those of our study possibly may be explained in part, by the use of a different serotype of LPS and/or by other undefined conditions.

Secondly, our data reveal that LPS-stimulated AM release LTB_4 and NAP-1 in sequence. LTB_4 release commenced first at $\sim$ 1 h and peaked by 3 h. The amount of LTB_4 present in supernatants of AM stimulated for 24 h was not significantly different from that quantitated in the supernatants of cells not purposefully stimulated. By contrast, LPS-elicited AM release of NAP-1 began between 3 and 5 h after challenge and was maximum at the last time point tested, 24 h (Fig. 2 *A*). We have shown previously by ELISA, HPLC-CM elution pattern, and NH_2-terminal sequence analysis that the NAP present in the 24-h supernatants of LPS-stimulated normal, adhered AM is identical to that released by normal human peripheral blood monocytes (27).

The time-response curve of LTB_4 release induced by zymosan was similar to that observed with LPS. Human monocytes possess a receptor for β-glucans (36). Zymosan activation of this receptor on human peripheral blood monocytes results in the generation and release of LTB_4 and LTC_4 (37). The active constituent of zymosan particles that elicits leukotriene release is β-glucan (36). Soluble β-glucans inhibit monocyte phagocytosis of zymosan and deminish LTB_4 release by these cells by $\sim$ 60–70% (37). We observed a similar inhibitory effect (mean of $\sim$ 40%, n = 4) by soluble β-glucans on LTB_4 release by AM stimulated with zymosan. The effect of treatment of AM with soluble β-glucans (laminarin) was specific for zymosan because laminarin did not inhibit AM release of LTB_4 induced by LPS. In our experiments zymosan also was a stimulus for AM release of NAP-1. Release of NAP-1 began at $\sim$ 5 h after challenge, a time when amounts of LTB_4 present were declining. Thus, activation of AM β-glucan receptors by zymosan elicits the sequential release of at least two neutrophil chemotaxins. AM release of these products in response to β-glucan mediated phagocytosis may provide the nonimmune host with the ability to initiate neutrophil activation and recruitment to the lung because β-glucan mediated phagocytosis occurs in the absence of opsonins (38).

The calcium ionophore A23187 is a carboxylic acid antibiotic which induces the influx of divalent cations across biologic membranes and the redistribution of intracellular calcium (39–41). Stimulation of human leukocytes with A23187 results in a Ca^{++}-dependent translocation of 5-lipoxygenase from the cytosolic compartment to a membrane-bound site

(42). In this location the 5-lipoxygenase is utilized for leukotriene synthesis. In contrast to AM release of LTB_4 and NAP-1 induced by either LPS or zymosan, the time course and profile of mediator release from AM in response to nonphysiologic stimulation with the calcium ionophore A23187 was distinctly different. A23187 induced a rapid rise in the release of LTB_4 that peaked by 30 min. Quantities of LTB_4 present in AM supernatants decreased at subsequent time points. Nevertheless, significant amounts of LTB_4 remained in AM supernatants harvested 24 h after stimulation (Fig. 4). Activation of calcium transmembrane fluxes by calcium ionophore bypasses ligand–receptor interactions but remains a sufficient stimulus for the proliferation of human peripheral blood lymphocytes (43) and the release by these cells of human immune interferon (44). Although A23187 is one of the most potent stimuli for leukotriene synthesis from numerous cell types, A23187 was not in our experiments an effective stimulus for NAP-1 release.

The LTB_4 time–release curves observed with LPS in this study demonstrated a decline in the concentration of LTB_4 by 24 h, as determined by both RIA and RP-HPLC (Fig. 1). Similarly, quantities of immunoreactive LTB_4 in supernatants of zymosan- or A23187-stimulated AM also declined by 24 h, Figs. 2 *B* and 4, respectively. These observations are explained by AM degradation of LTB_4 (Fig. 6). Our results are consistent with those of Schönfeld and co-workers (45) who observed that human lung macrophages obtained from minced fragments of surgical specimens of patients with bronchial carcinoma partially metabolized LTB_4 after 1 h to products that are distinct from LTB_4 (45). These products were completely resolved by RP-HPLC not only from LTB_4 but also from its 20-OH- and 20-COOH-LTB_4 ω-oxidation metabolites, which result from neutrophil metabolism of LTB_4. Furthermore, these metabolites were not recognized by LTB_4 antisera, and they did not absorb at 280 nm (45). Their data suggest that at least one of these metabolites appeared to be a reduced form of LTB_4, 5,12-dihydroxyeicosatrienoic acic (45). This product was described originally as a novel metabolite of rat mesangial cells and mouse bone marrow-derived macrophages by Kaever and colleagues (46, 47). In our experiments analysis by RP-HPLC of supernatants from adhered AM cultured without LPS for significantly longer than 1 h, namely 24 h, in the presence of a trace amount of [^{3}H]LTB_4 revealed five radiolabeled peaks that were clearly separated from LTB_4 by RP-HPLC. Resolution by RP-HPLC of the supernatants from AM cultured in an identicle manner but with LPS demonstrated these same five peaks and only one additional metabolite. Peak 1, which was observed in the culture media that was incubated for 24 h without AM, was found in greater proportions in supernatants from AM cultures. This peak coeluted with the two ω-oxidation products of LTB_4. AM cultures prepared as described in our methods routinely contain $> 95\%$ macrophages and $< 0.5\%$ neutrophils. However, we cannot rule out the possibility that the small numbers of cells that contaminated the AM cultures, or the macrophages themselves, may have contributed to the metabolism of LTB_4 over the 24 h in culture. Thus, our results extend those of Schönfeld and co-workers (45) by revealing that human alveolar macrophages from normal subjects also metabolize LTB_4. The precise identity of these metabolites has not been determined. Additional experiments will be needed to identify and functionally evaluate these novel macrophage products. For example, we have not determined whether or not the metabolites of LTB_4 present in 24-h supernatants might contribute to the overall chemotactic activity observed. In this regard Kaever and colleagues (47) have determined that dihydro-LTB_4 is significantly less chemotactic for human leukocytes than is LTB_4.

Thirdly, our data offer at least a partial explanation for previous and discrepant observations by others on human AM-derived neutrophil chemotaxins elicited in vitro. Merrill and colleagues (48) found neutrophil chemotaxins with molecular weights of about 10,000 and $< 1{,}000$ daltons in 24-h culture fluids of human AM. Based on our data, the larger molecule was probably NAP-1. The smaller molecule, which was detected in concentrated fluids, may have been a small amount of LTB_4 still present at the 24-h time point. The unidentified lipid molecule found by Hunninghake and colleagues (49) in culture fluids of AM stimulated with a variety of physiologically relevant stimuli may also have been LTB_4. Recently, Martin and colleagues (13) concluded that LTB_4 is the predominant chemotaxin in culture fluids of human AM stimulated with A23187 or opsonized zymosan. Our results agree with their A23187 data, since the calcium ionophore did not stimulate NAP-1 secretion. One possible explanation for the fact that Martin and colleagues (13) did not detect NAP-1 in supernatants of AM incubated with NDGA and stimulated for 24 h with opsonized zymosan may be explained by our observation that NDGA at the concentrations used in both our studies, 10^{-4} M, completely blocks not only LTB_4 release but also NAP-1 release (Fig. 5).

We believe for several reasons that it is premature to conclude that LTB_4 is the only important neutrophil chemotaxin with functional activity released from AM at early time points and that NAP-1 is the only important neutrophil chemotaxin present at 24 h. First, AM cultured in vitro and purposefully stimulated also release 5-HETE (9, 10, 12). We did not assess our supernatants for the presence of this product. Secondly, adhered AM from normal subjects release an inhibitor of neutrophil chemotaxis that can be detected in supernatants as little as thirty minutes after cultures are established (50). Thirdly, we showed previously that NAP-1 does not account for all the chemotactic activity in 24-h culture fluids of LPS-stimulated AM (27). Thus, the neutrophil chemotactic activity time curve that results from AM stimulated in vitro is most likely the result of the cumulative effects of many factors that are present in AM supernatants at varying concentrations and at varying time points.

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