

Lipopolysaccharides Prime Whole Human Blood and Isolated Neutrophils for the Increased Synthesis of 5-Lipoxygenase Products by Enhancing Arachidonic Acid Availability: Involvement of the CD14 Antigen

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Summary

Stimulation of heparinized blood with 1 μ M formyl-methionyl-leucyl-phenylalanine (FMLP) resulted in the formation of <30 pmol/ml plasma of 5-lipoxygenase (5-LO) products. The preincubation of blood with 1 μ g/ml of lipopolysaccharide (LPS) (*Escherichia coli* 0111-B4) for 30 min before stimulation with FMLP resulted in the accumulation of 250–300 pmol of 5-LO products per ml plasma. The major products detected were leukotriene B₄ and (5S)-hydroxy-6, 8, 11, 14-eicosatetraenoic acid which were produced in equivalent amounts. The priming activity was detectable with as little as 1–10 ng LPS per ml blood and was optimal using 1–10 μ g LPS/ml blood. The priming for 5-LO product synthesis was optimal after 20–30 min of preincubation with LPS and declined at preincubation times >30 min. The priming effect of LPS was also observed using the complement fragment C5a or interleukin 8 as agonists. Polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells accounted for 80 and 20% of the synthesis of 5-LO products, respectively. The ability of LPS to prime isolated PMN was dependent on the presence of plasma and was inhibited by the anti-CD14 antibody IOM2, indicating a CD14-dependent priming mechanism. The priming of whole blood with tumor necrosis factor α (TNF- α) and LPS was additive and the presence of mononuclear cells did not enhance the ability of LPS to prime PMN, indicating that the priming activity of LPS is independent of LPS-induced TNF- α synthesis. The mechanism by which LPS enhance 5-LO product synthesis in PMN was investigated. Treatment of PMN with LPS strongly enhanced the release of arachidonic acid after stimulation with FMLP. The release of arachidonic acid was optimal 2–3 min after stimulation with FMLP, attaining levels 5–15-fold greater than those observed in unprimed cells stimulated with FMLP. These results demonstrate that LPS dramatically increases the ability of blood to generate 5-LO products, and support the putative role of leukotrienes in pathological states involving LPS.

The brisk inflammatory reaction which occurs in response to gram-negative pathogens is largely induced by LPS (or endotoxin), which directly stimulate leukocytes for a host of defensive responses (1–5). Indeed, when exposed to LPS, leukocytes release prostaglandins (6), exhibit increased expression of CD11b/CD18 and adhesion (7–9), and produce cytokines such as IL-1 (10) and TNF- α (11). Additionally, LPS prime leukocytes in vitro for an increased capacity for several functional responses, including arachidonate release from macrophages (12, 13), as well as leukotriene (14), platelet-activating factor (15), and superoxide production (16–18) by neutrophils. In localized infections, LPS are largely restricted to inflammatory sites, enhancing host defense. However, if the infection is not brought under control, endotoxin and/or inflammatory mediators may reach the circulation, predisposing the

microvasculature to thrombosis (2) and can lead to systemic endotoxemia or sepsis and associated complications including septic shock (19), adult respiratory distress syndrome (ARDS) (20), and multiorgan failure (21).

Leukotriene B₄ (LTB₄)¹, one of the most potent inflammatory chemoattractants (22, 23), and other arachidonic acid (AA) metabolites such as (5S)-hydroxy-6,8,11-14-eicosatetraenoic acid (5-HETE) are produced by the 5-lipoxygenase (5-LO) pathway of neutrophils and monocytes-macrophages upon

¹ Abbreviations used in this paper: AA, arachidonic acid; 5-HETE, (5S)-hydroxy-6,8,11,14-eicosatetraenoic acid; 5-LO, 5-lipoxygenase; LBP, LPS binding protein; LT, leukotriene; LTB₄, leukotriene B₄; PGB, prostaglandin B₂.

stimulation with a variety of agonists. Neutrophils, which are the primary effector cells in inflammation, are also intimately involved in the pathogenesis of sepsis and associated complications (2, 19, 21, 24, 25). Likewise, 5-LO products have been implicated in LPS-induced endotoxic shock, pulmonary granulocyte sequestration, and pulmonary dysfunction and liver damage (26–30). Although neutrophils and AA metabolites from the 5-LO pathway are implicated in the defensive inflammatory responses to bacteria, as well as in the complications resulting from uncontrolled infections, there is limited information concerning the effects of bacterial LPS on the metabolism of 5-LO products in these cells. Whereas LPS do not directly stimulate neutrophils for the synthesis of 5-LO products, LPS have been shown to prime isolated neutrophils for an enhanced synthesis of LTB₄ in response to subsequent stimuli like the ionophore A23187, phorbol ester, or zymosan, but not to receptor-mediated stimuli (14). Similarly, mouse peritoneal macrophages and macrophage-like P388D₁ cells primed with LPS release more AA metabolites after stimulation than unprimed cells (12, 13).

The induction of several leukocytic responses by LPS recently has been shown to require the presence of the plasma proteins LPS binding protein (LBP) or sepsin which form complexes with LPS that are recognized by the cellular CD14 surface antigen (31–33). Importantly, these plasma proteins have been shown to be obligatory for LPS-induced leukocyte responses such as superoxide anion production, cell adhesion, integrin expression, and TNF- α secretion (7, 9, 18, 31, 33, 34). Few reports describe leukotriene (LT) synthesis in the presence of plasma or in blood (35) and none investigates the effect of LPS on LT synthesis in such conditions. The involvement of plasma proteins in cellular responses to LPS, coupled with the potentially complex dynamics of AA metabolism *in vivo* where transcellular synthesis of AA metabolites occurs (35–38), and where plasma may also affect AA metabolism (39, 40), underscores the importance of the study of the effects of LPS on lipid mediator synthesis in a physiologically relevant environment.

Human whole blood has been successfully used as an *in vivo* model for local cytokine production (41–43) and AA metabolism (35, 44). The use of this readily available intact human tissue reduces potential artifacts arising from cellular isolation procedures (45) and provides a physiological environment where cellular interactions are preserved. The present study characterizes the priming of leukocytes by LPS for the subsequent synthesis of 5-LO products in whole blood stimulated with soluble agonists.

Materials and Methods

Materials. Biosynthetic recombinant human TNF- α and GM-CSF were a gift from the Genetics Institute (Cambridge, MA). Recombinant human IL-8 was a gift from Dr. Henry Showell (Pfizer Pharmaceuticals, Groton, CT). LPS (*Escherichia coli* 0111-B4) was obtained from Difco Laboratories (Detroit, MI) and HBSS from GIBCO BRL (Burlington, Ont., Canada). Purified anti-CD14 mAb (IOM2) was obtained from AMAC Inc., (Westbrook, ME). The calcium ionophore A23187, recombinant human C5a, FMLP, and prostaglandin B₂ (PGB₂) were obtained from Sigma Chemical

Company (St. Louis, MO). AA and deuterated- (D₈) AA were obtained from Cayman Chemical Co. (Ann Arbor, MI). 19-hydroxy-PGB₂ was prepared as previously described (46). TNF- α , GM-CSF, and IL-8 were dissolved in nonpyrogenic water containing 0.01% BSA (low endotoxin), under sterile conditions, and stored at –20°C. LPS was dissolved in 0.9% NaCl. A23187 and FMLP were prepared in DMSO which had been filtered on alumin. Lyophilized C5a was reconstituted at 1 mg/ml in nonpyrogenic water. Ficoll-Paque (Pharmacia, Uppsala, Sweden) and water used for the preparation of various solutions were tested for endotoxin content by the *Limulus* Amebocyte Lysate Assay (Whittaker Bioproducts, Walkersville, MD) and found to contain endotoxin in the low pg/ml levels. All solutions and reagents used for cell preparation and priming were sterile.

Blood Incubations. Venous blood was obtained from healthy donors and collected into 10-cc glass tubes (100 × 16 mm Vacutainer; Becton Dickinson & Co., Mountain View, CA) containing 143 USP units of heparin. All donors had normal differential leukocyte counts. Heparinized blood was preincubated in the presence of LPS or its diluent at 37°C for 30 min unless otherwise indicated. The blood was then dispensed in polypropylene tubes (0.5 ml per tube) and incubated in a shaking water bath at 37°C for 15 min in the presence of DMSO (0.2%), FMLP, C5a, or IL-8. Incubations were stopped at 0°C (ice-water bath) and blood samples were immediately centrifuged at 400 *g* for 3 min at 4°C. A 200- μ l aliquot of plasma was collected from each sample and added to 5 vol of acetonitrile/methanol (1:1, vol/vol) containing 12.5 ng each of PGB₂ and 19-hydroxy-PGB₂ as internal standards. Protein precipitation was achieved at –20°C overnight before analysis of 5-LO products by HPLC (44).

Isolated Cell Preparations. PMN and PBMC were isolated from peripheral blood after dextran sedimentation and centrifugation on Ficoll-Paque cushions as previously described (47). Leukocyte-depleted blood was obtained by removal of the buffy coat after four successive centrifugations of whole blood and resuspensions of the sedimented RBC with autologous plasma. The final RBC preparation containing <5% of the normal blood leukocytes was resuspended in autologous plasma (1:1, vol/vol) and referred to as leukocyte-depleted blood. In some experiments, PMN and/or PBMC were resuspended at 5 × 10⁶ and 3.5 × 10⁶ cells, respectively, per ml of autologous leukocyte-depleted blood.

Blocking Studies with Anti-CD14. For blocking studies, 5 × 10⁶ PMN were suspended in 200 μ l of calcium-free HBSS containing (or not) 5 μ g of purified IOM2 anti-CD14 mAb, which was previously shown to inhibit the specific binding of LPS to monocytes (48), or 5 μ g of an isotype control antibody for 30 min at 4°C. The cells were then washed and resuspended in HBSS supplemented with 10% heat-inactivated autologous plasma and incubated in the presence or absence of LPS (1 μ g/ml) or GM-CSF (1 nM) for 30 min at 37°C. The cells were washed once by centrifugation, resuspended in HBSS, and stimulated with FMLP (0.1 μ M) for 10 min. Reactions were stopped by the addition of 1 vol of organic solvents containing standards (see blood incubations above) and 5-LO products were analyzed as described below.

Analysis of 5-Lipoxygenase Products. The denatured samples (plasma or isolated leukocyte suspensions) were centrifuged at 2,000 *g* for 20 min to remove the precipitated material, the supernatants were diluted to 75% water and analyzed without further treatment by reverse phase (RP) HPLC using an on-line extraction procedure as described previously (44, 46).

Analysis of Free Arachidonic Acid in Isolated PMN. Isolated neutrophils were preincubated for 30 min at 37°C in HBSS containing 10% plasma with or without 1 μ g/ml LPS. After the preincuba-

tion period, cells were washed by centrifugation and resuspended in plasma-free HBSS. Cells were then stimulated with 0.1 μM FMLP at 37°C and at various times the incubations were terminated by the addition of 2 vol of ice-cold methanol containing 20 ng of D₈-AA as an internal standard. Samples were processed as described above for HPLC analysis and the HPLC fractions containing AA (determined by using a ³H-AA standard) were collected. The samples were evaporated under reduced pressure (using a Speed Vac model SVC 100D; Savant Instruments Inc., Farmingdale, NY) and redissolved into 100 μl of acetonitrile. AA was assayed by liquid chromatography-mass spectrometry (LC-MS), using a nebulizer-assisted electrospray (ion spray) interface coupled to a triple-quadrupole MS (API-III; PE Sciex, Thornhill, Ont., Canada). 6 μl aliquots of the samples were injected into the electrospray interface via the 20- μl loop of a Rheodyne injector (model 9125; Rheodyne, Cotati, CA) connected to a short column (2 \times 30 mm, packed with 5 μm C₁₈ particles), using acetonitrile:H₂O (87.5:12.5, vol/vol, containing 0.1% acetic acid) as solvent, at a flow rate of 150 $\mu\text{l}/\text{min}$. Samples were analyzed in the negative ion mode. The ions at *m/z* 303 and 311, representing the carboxylate anions of AA and D₈-AA, respectively, were monitored.

Results

LPS Priming of Blood for the Synthesis of 5-LO Products: Kinetics and Dose-Response. After preincubation with LPS, the stimulation of blood with 1 μM FMLP resulted in the synthesis of 5-HETE, LTB₄ and the ω -oxidation products of LTB₄, 20-hydroxyl-LTB₄ (20-OH-LTB₄), and 20-COOH-LTB₄, as shown on the HPLC-chromatograms (Fig. 1). In the absence of LPS, stimulation of blood with FMLP resulted in a much smaller yet detectable synthesis of 5-LO products.

The priming effect of LPS was rapid, maximal capacity for the synthesis of 5-LO products being observed after 30 min of preincubation of blood with 1 μg LPS/ml (Fig. 2). The synthetic capacity of blood rapidly decreased at longer preincubation times, and the priming effect of LPS was no longer detectable at 90–240 min (data not shown). The preincubation of blood in the absence of added LPS, followed by stimulation with FMLP, resulted in little or no significant synthesis of 5-LO products at any of the time intervals tested. When blood was preincubated for 30 min with varying concentrations of LPS before stimulation with 1 μM FMLP, a priming effect for the synthesis of 5-LO products was observed with as little as 1–10 ng LPS per ml of blood. The maximal priming response was obtained in samples preincubated with 1 μg LPS per ml of blood (Fig. 3). The preincubation of blood with LPS, without subsequent FMLP stimulation, resulted in little if any synthesis of 5-LO products except at the higher LPS concentrations (1–10 $\mu\text{g}/\text{ml}$) where LPS alone induced the synthesis of LTB₄ in amounts equivalent to ~5% of the amount synthesized after FMLP stimulation of LPS-treated blood (data not shown).

The predominant 5-LO products detected in blood primed with LPS and stimulated with FMLP were LTB₄ and 5-HETE which were synthesized in approximately equivalent amounts. Typically, 125–150 pmol of each compound was detected per ml of plasma obtained from blood primed with 1 μg LPS for 30 min and subsequently stimulated for 15 min with 1 μM FMLP. However, the magnitude of the

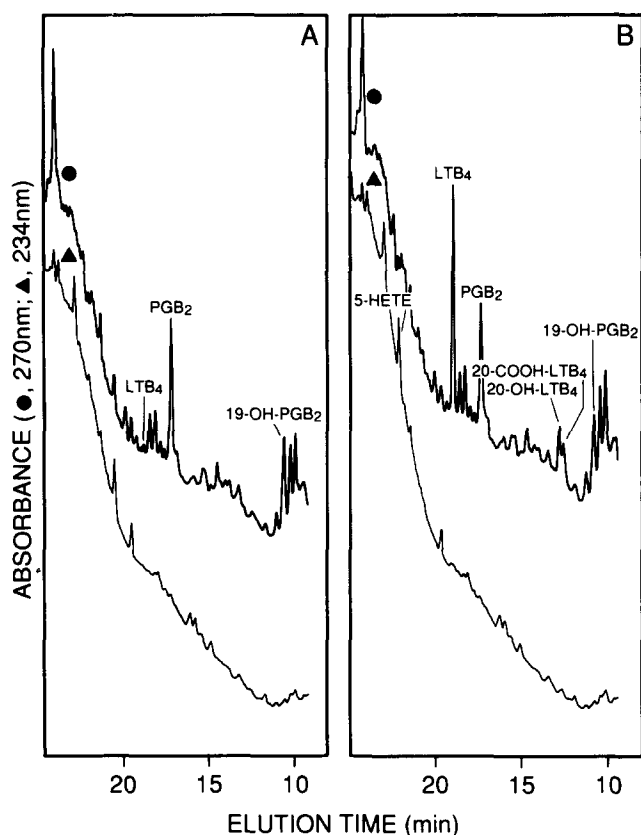


Figure 1. RP-HPLC chromatograms of eicosanoids in plasma samples. Heparinized blood aliquots were preincubated for 30 min at 37°C with 1 $\mu\text{g}/\text{ml}$ LPS (B) or its diluent (0.9% NaCl) (A) before stimulation for 15 min with 1 μM FMLP. Plasma samples (200 μl) were taken and processed for analysis by RP-HPLC as described in Materials and Methods. The amount of internal standards added per sample was 12.5 ng each of PGB₂ and 19-OH-PGB₂, and 80% of each sample was injected. Attenuation settings of the UV photodiode array detector were 0.01 and 0.05 absorbance units at full scale at 270 and 234 nm, respectively.

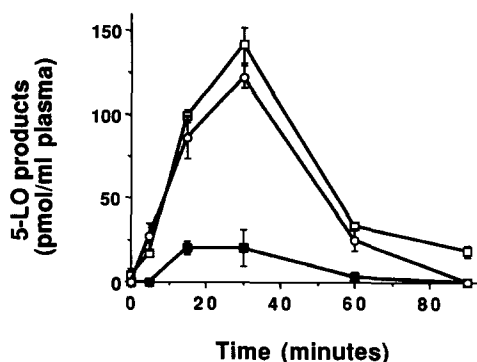


Figure 2. Kinetics of the priming effect of LPS on the synthesis of LTB₄ (open squares), its ω -oxidation products 20-OH- and 20-COOH-LTB₄ (filled squares), and 5-HETE (open circles) in whole blood stimulated with FMLP. Heparinized human blood was preincubated with LPS (1 $\mu\text{g}/\text{ml}$) for various periods of time before stimulation for 15 min with 1 μM FMLP. 5-LO products were analyzed by RP-HPLC as described in Materials and Methods. Values are the means \pm SD from one experiment performed in triplicate which is representative of four separate experiments.

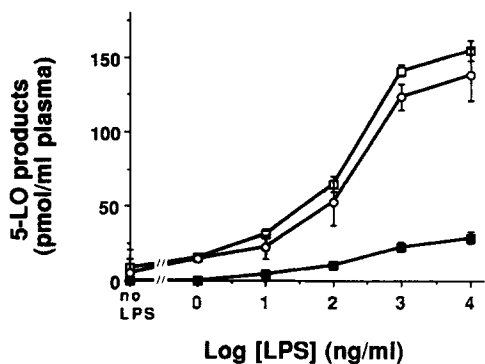


Figure 3. The dose-response of the priming effect of LPS on the synthesis of LTB₄ (open squares), its ω -oxidation products 20-OH- and 20-COOH-LTB₄ (filled squares), and 5-HETE (open circles) in whole blood stimulated with FMLP. Heparinized human blood was preincubated with the indicated concentrations of LPS for 30 min before stimulation for 15 min with 1 μ M FMLP. 5-LO products were analyzed by RP-HPLC as described in Materials and Methods. Values are the means \pm SD from one experiment performed in triplicate which is representative of six experiments.

response ranged from 50 to 200 pmol of LTB₄ or 5-HETE per ml of plasma, depending on the donor. The ω -oxidation metabolites of LTB₄, 20-OH-LTB₄, and 20-COOH-LTB₄, were detected in amounts representing \sim 10–20% of the quantity of LTB₄ measured, independently of the incubation time with FMLP.

Effect of LPS on the Synthesis of 5-LO Products Induced by Various Agonists. The chemotactic formylated bacterial tripeptide, FMLP, was a more potent agonist for the stimulation of 5-LO products in LPS-primed blood than were the human complement fragment C5a or the chemoattractant IL-8 (Fig. 4). In dose-response studies, optimal synthesis of 5-LO products in blood primed with 1 μ g LPS/ml was obtained with 0.5 μ M of human C5a (data not shown). However, this only

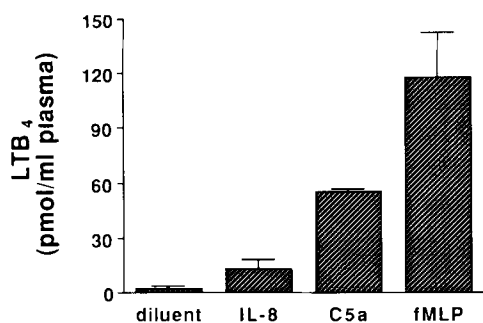


Figure 4. Effect of FMLP, C5a, or IL-8 on the synthesis of LTB₄ in LPS-treated whole blood. Heparinized human blood was preincubated with 1 μ g/ml LPS for 20 min before stimulation for 15 min with 1 μ M FMLP, 0.5 μ M C5a, 0.5 μ M IL-8, or their diluents. LTB₄ and its ω -oxidation metabolites were analyzed by RP-HPLC as described in Materials and Methods. Values are the means of three separate experiments each performed in triplicate \pm SEM and represent the total amount of LTB₄ and its ω -oxidation metabolites. The values in all columns are significantly different from one another as determined by analysis of variance using Fisher's protected least significant difference, $p < 0.05$.

resulted in half the synthetic capacity observed when using 1 μ M FMLP as the stimulus. Stimulation of LPS-primed blood with the highest concentration of IL-8 tested, 0.5 μ M, only resulted in a response equivalent to \sim 10% of that observed with FMLP stimulation.

Contribution of PMN and Mononuclear Cells in the LPS Priming of Blood. As indicated in Fig. 5, \sim 80% of the 5-LO products detected in LPS-primed blood are released by PMN whereas the remaining 20% of the activity is found in the PBMC fraction. This was determined by adding either isolated PMN or PBMC, in physiological concentrations, to autologous leukocyte-depleted blood. Leukocyte-depleted blood alone, when preincubated with 1 μ g/ml LPS, did not synthesize any detectable 5-LO products after stimulation with 1 μ M FMLP. Importantly, when both PMN and PBMC were added to the leukocyte-depleted blood, the capacity for the synthesis of 5-LO products resulting from a preincubation with LPS was equal to the sum of the synthetic capacities of blood partially reconstituted with the two leukocyte populations separately.

Dependence on Plasma and CD14 for LPS Priming Activity. The requirement for plasma in the priming effect of LPS for the synthesis of 5-LO products was determined using isolated PMN. PMN were isolated and preincubated with various concentrations of LPS in HBSS alone or in HBSS supplemented with 10% plasma, for 30 min at 37°C. After the preincubation period, the cells were washed by centrifugation, resuspended in HBSS, and stimulated for 10 min with 0.1 μ M FMLP. Fig. 6 demonstrates that the ability of LPS to prime neutrophils for an enhanced synthesis of 5-LO prod-

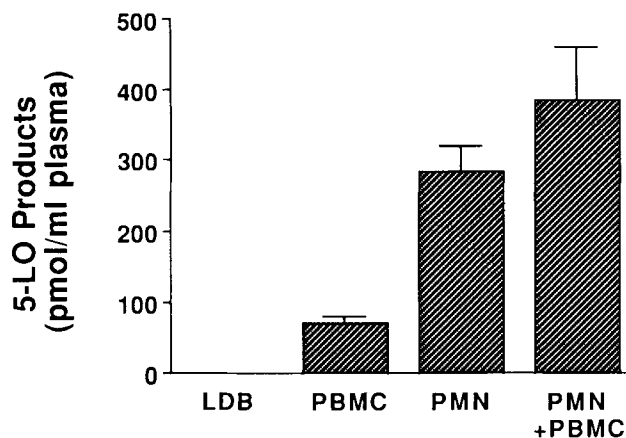


Figure 5. The priming by LPS of leukocyte-depleted human blood (LDB) or leukocyte-depleted blood partially reconstituted with the PBMC fraction (3.5×10^6 cells/ml), the PMN fraction (5×10^6 cells/ml), or both fractions (PMN+PBMC), for the synthesis of 5-LO products following stimulation with FMLP. The different fractions of blood were preincubated with 1 μ g LPS/ml for 20 min at 37°C before stimulation for 15 min with 1 μ M FMLP. 5-LO products were analyzed by RP-HPLC as described in Materials and Methods. Values are the means \pm SD from one experiment performed in triplicate which is representative of three separate experiments. Values represent the total amount of LTB₄, its ω -oxidation metabolites, and 5-HETE.

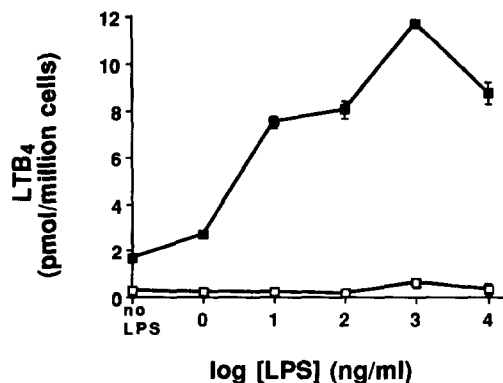


Figure 6. Effect of plasma on the priming of PMN with LPS. PMN were isolated and preincubated for 30 min (at 10^7 cells/ml) with $1 \mu\text{g/ml}$ LPS at 37°C in the absence (open squares) or presence (filled squares) of 10% autologous plasma in HBSS. The cells were then washed by centrifugation, resuspended in 0.5 ml HBSS (at 10^7 cells/ml), and stimulated for 10 min with $0.1 \mu\text{M}$ FMLP. 5-LO products were analyzed by RP-HPLC as described in Materials and Methods. Values are the means \pm SD from one experiment performed in triplicate which is representative of three separate experiments.

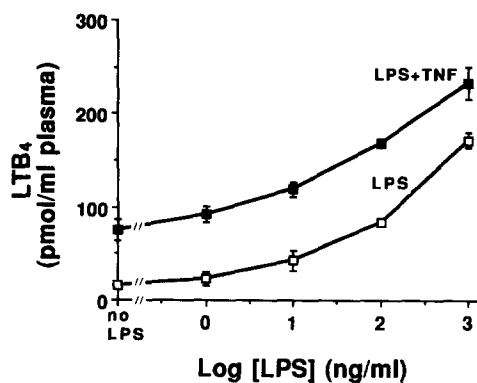


Figure 8. Additivity of the priming effect of LPS and $\text{TNF-}\alpha$ in blood. Heparinized human blood was preincubated with the indicated concentrations of LPS with or without 1.2 nM $\text{TNF-}\alpha$ for 30 min before stimulation for 15 min with $1 \mu\text{M}$ FMLP. 5-LO products were analyzed by RP-HPLC as described in Materials and Methods. Values are the means \pm SD from one experiment performed in triplicate which is representative of four separate experiments and represent the total amount of LTB_4 and its ω -oxidation metabolites.

ucts in response to FMLP was dependent on the presence of plasma during the preincubation period.

The dependence on plasma for the priming effects of LPS on 5-LO product synthesis suggested the involvement of interactions with the CD14 antigen. Therefore, the ability of the anti-CD14 mAb, IOM2, to block the priming activity of LPS was assessed. When the IOM2-treated PMN were incubated with $1 \mu\text{g/ml}$ LPS, their response to FMLP stimulation was significantly decreased compared with cells preincubated in the absence of IOM2 (Fig. 7). In contrast, preincubation with the antibody did not block the priming activity

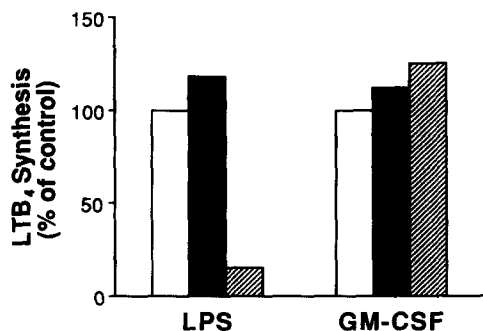


Figure 7. Effect of the anti-CD14 mAb (IOM2) on the priming effect of LPS and of GM-CSF on LTB_4 synthesis by FMLP-stimulated PMN. PMN were isolated and incubated on ice for 30 min with no addition (open bars), with IOM2 ($1 \mu\text{g}/10^6$ cells) (stippled bars), or with an IgG2a isotype control ($1 \mu\text{g}/10^6$ cells) (filled bars). Cells were then incubated for 30 min with $1 \mu\text{g/ml}$ LPS or 1.0 nM GM-CSF in 10% autologous plasma at 37°C , washed, and then stimulated for 10 min with $0.1 \mu\text{M}$ FMLP. 5-LO products were analyzed by RP-HPLC as described in Materials and Methods. Values are presented as the percent synthesis of LTB_4 (and its ω -oxidation metabolites) compared to incubations in the absence of antibody (control). Values are from one experiment performed in duplicate which is representative of three separate experiments.

of GM-CSF, which is known to prime the cells via its own receptor, and the priming activity of LPS was not affected by the preincubation of cells with isotype control antibodies.

LPS Priming Activity in Whole Blood: Comparisons with $\text{TNF-}\alpha$. The potential role of LPS-induced $\text{TNF-}\alpha$ production in the priming of blood by LPS for the synthesis of 5-LO products was investigated. In a first series of experiments, the priming activity of LPS and $\text{TNF-}\alpha$ were compared. The preincubation of blood with $1 \mu\text{g}$ LPS/ml routinely resulted in a two- to fourfold greater synthesis of 5-LO products after stimulation with FMLP than that observed with blood pretreated with $\text{TNF-}\alpha$ under optimal conditions (1.2 nM , 20 min, data not shown). These results indicate

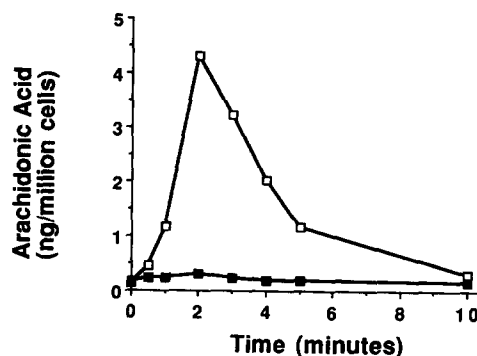


Figure 9. Effect of priming with LPS on the level of free AA in PMN suspensions. PMN were isolated and preincubated for 30 min with $1 \mu\text{g/ml}$ LPS (open squares) or diluent (filled squares) at 37°C in HBSS containing 10% autologous plasma. The cells were then washed by centrifugation, resuspended at a density of 10^7 cells/ml in HBSS, and 0.5 ml aliquots of the cell suspensions were stimulated with $0.1 \mu\text{M}$ FMLP. Reactions were stopped with 3 vol of methanol (0°C) containing 20 ng of deuterated (D8)-AA as an internal standard. Samples were subjected to HPLC and fractions containing AA were collected for quantitation by mass spectrometry. Values are the means from one experiment performed in duplicate which is representative of four separate experiments.

that LPS-induced synthesis of TNF- α alone is not responsible for the observed priming activity of LPS in blood.

To determine possible interactions between LPS and TNF- α in the observed priming effect, blood was primed with a range of LPS concentrations alone or in combination with TNF- α (1.2 nM). At all concentrations of LPS tested, coincubation with TNF- α resulted in a priming activity equivalent to the sum of that of TNF- α alone and LPS alone (Fig. 8). This suggests that LPS and TNF- α are priming blood via different mechanisms and further indicates that TNF- α production was not involved in the priming activity of LPS for the synthesis of 5-LO products in whole blood.

Effect of LPS Priming on Free AA Levels in Stimulated PMN. Levels of free AA were determined by mass spectrometric analysis in LPS-primed and unprimed PMN after stimulation with FMLP. Fig. 9 demonstrates that the levels of free AA in LPS-primed cells increase significantly within 30 s after stimulation with FMLP attaining maximal levels 2–3 min after stimulation. The peak in AA levels obtained varied from donor to donor, reaching levels from 5–15-fold above baseline. In contrast, unprimed cells only show a minor increase in AA levels after stimulation with FMLP. In the absence of stimulation, the basal levels of free AA were the same in primed and unprimed cells, indicating that LPS alone did not induce an increase in the levels of free AA.

Discussion

Bacterial LPS prime leukocytes for enhanced functional capacities (15, 16, 17, 49), including AA metabolism (12–14, 50). Because of the already accumulated evidence for a role of leukotrienes in pathological states related to sepsis, and given the recent demonstration that plasma proteins are required for the stimulation by LPS of functional responses by leukocytes, we sought to study the effects of LPS on the production 5-LO products in whole blood *ex vivo*, a model relevant to physiological and pathophysiological conditions.

The data reported herein demonstrate that LPS efficiently prime whole blood *ex vivo* for the subsequent synthesis of LTB₄ and 5-HETE after stimulation with the receptor-mediated soluble agonists FMLP, C5a or IL-8. This is in contrast to the reported lack of priming effect of LPS in isolated neutrophils stimulated with FMLP in protein-free buffer (14). Similar to the priming effect of LPS on O₂⁻ release (8) and to the direct effect of LPS on CR3 activity in PMN (7), the priming effect of LPS on 5-LO product synthesis was transient.

The synthesis of 5-LO products induced by FMLP in LPS-primed blood was compared with that induced by C5a and IL-8 which are also known to activate human neutrophil 5-LO (51, 52) and which are implicated in the pathophysiological response to LPS (1, 53). IL-8 (0.5 μ M) was a weak agonist for 5-LO product synthesis in whole blood although the concentration used was relatively elevated compared with concentrations required for optimal biological activity in isolated neutrophils. The binding of IL-8 to erythrocytes and to circulating anti-IL-8 IgG in plasma were recently reported and may be mechanisms by which circulating IL-8 is neutralized

(54, 55). However, the concentrations of IL-8 used in the present study far exceed the reported binding capacities of erythrocytes or anti-IL-8 IgG. Consequently, these binding activities do not explain the relatively low activity for IL-8 reported in this model.

An important difference in the LPS priming of whole blood compared with isolated cells may be the participation of plasma proteins in the binding of LPS to leukocytes. Numerous leukocytic cell-surface proteins have been described which bind LPS (56). One cell-surface protein whose interaction with LPS invokes cellular responses is the CD14 antigen which was first described as a monocyte/macrophage differentiation antigen (32). LPS binding to CD14 requires the presence of plasma since the ligand for CD14 is actually a complex of LPS with plasma proteins. Two separate proteins, LBP and sepsin, have been identified which, when complexed to LPS, form the ligand for CD14 (31, 33). The priming of PMN, which represent 85% of the CD14-positive cells in blood as determined by cytofluorometric analysis (data not shown), was dependent on plasma and was blocked by pretreatment of cells with an anti-CD14 blocking antibody indicating that the effect of LPS observed here is mediated via the CD14 receptor. Additionally, preincubation of PMN with the antibody had no effect on the synthesis of 5-LO products when cells were subsequently primed with GM-CSF and stimulated with FMLP, indicating that the antibody specifically inhibited the cellular response to LPS and not the ability to synthesize 5-LO products, nor the response to FMLP.

Pathologies associated with bacteremia have been suggested to result at least in part from LPS-induced TNF- α production (57). Monocytes or macrophages incubated in the presence of LPS secrete important quantities of TNF- α which itself can activate or prime cells for enhanced cellular functions including LTB₄ synthesis (58). This raises the possibility that the priming effect observed in the present study on the synthesis of 5-LO products may be mediated by LPS-induced TNF- α synthesis. Several observations, however, argue against this hypothesis. The finding that the priming activity of optimal concentrations of TNF- α is additive with various concentrations of LPS suggests that LPS prime blood for the synthesis of 5-LO products independently of TNF- α . An increase in the TNF- α concentration in plasma is only detected 1 h after the treatment of blood with LPS, with maximal production at 4–6 h (41, 42), whereas LPS priming of 5-LO product synthesis is optimal within 30 min. Furthermore, the priming effect of LPS in blood enables the synthesis of 5-LO products which is of severalfold greater magnitude than that achieved with optimal concentrations of TNF- α (1.2 nM) (data not shown). Finally, the presence of mononuclear cells, which are the principle cells responsible for cytokine synthesis in blood (including TNF- α), did not affect LPS priming of PMN, suggesting that LPS is acting directly on granulocytes with no contribution from monocyte secretory products. LPS priming of isolated PMN further supports this hypothesis.

The mechanisms by which LPS or cytokines prime leukocytes for an increased synthesis of 5-LO products have not

yet been fully elucidated. An enhanced synthesis of 5-LO products may result from an increased activity of the 5-LO enzyme and/or from increased availability of free arachidonate. It must be stressed, however, that in PMN stimulated by soluble agonists such as FMLP, C5a, or PAF, the synthesis of 5-LO products is primarily dependent on AA availability as demonstrated in experiments where the addition of exogenous substrate leads to a dramatic increase in 5-HETE and LT synthesis (51, 52, 59–61). Accordingly, in previous studies increases in AA levels were observed in GM-CSF-primed PMN stimulated with PAF (62) and TNF- α was shown to prime PLA₂ activation in neutrophils (58). In the present study,

measurement of AA by mass spectrometry demonstrated striking differences in free AA levels between LPS-primed cells and unprimed cells after FMLP stimulation. Such data strongly suggest that enhanced synthesis of 5-LO products in LPS-primed PMN may be explained on the basis of increased substrate availability. Nevertheless, the possible contribution of an enhanced ability to transform the free AA (i.e., an increased 5-LO activity) cannot be ruled out. Studies are now in progress to elucidate the mechanism by which LPS prime PMN for enhanced AA availability and to assess the possible actions of LPS at the level of the 5-LO enzyme and of its activation process.

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