Neutrophils Exposed to Bacterial Lipopolysaccharide Upregulate NADPH Oxidase Assembly

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Abstract

Bacterial LPS is a pluripotent agonist for PMNs. Although it does not activate the NADPH-dependent oxidase directly, LPS renders PMNs more responsive to other stimuli, a phenomenon known as "priming." Since the mechanism of LPS-dependent priming is incompletely understood, we investigated its effects on assembly and activation of the NADPH oxidase. LPS pretreatment increased superoxide (O_2^-) generation nearly 10-fold in response to *N*-formyl methionyl leucyl phenylalanine (fMLP). In a broken-cell O_2^--generating system, activity was increased in plasma membrane–rich fractions and concomitantly decreased in specific granule–rich fractions from LPS-treated cells. Oxidation-reduction spectroscopy and flow cytometry indicated LPS increased plasma membrane association of flavocytochrome b_{558}. Immunoblots of plasma membrane vesicles from LPS-treated PMNs demonstrated translocation of p47-*phox* but not of p67-*phox* or Rac2. However, PMNs treated sequentially with LPS and fMLP showed a three- to sixfold increase (compared with either agent alone) in plasma membrane–associated p47-*phox*, p67-*phox*, and Rac2, and translocation paralleled augmented O_2^- generation by intact PMNs. LPS treatment caused limited phosphorylation of p47-*phox*, and plasma membrane–enriched fractions from LPS- and/or fMLP-treated cells contained fewer acidic species of p47-*phox* than did those from cells treated with PMA. Taken together, these studies suggest that redistribution of NADPH oxidase components may underlie LPS priming of the respiratory burst. (*J. Clin. Invest.* 1998. 101:455–463.) Key words: inflammation • superoxide • endotoxin • respiratory burst • polymorphonuclear leukocytes

Introduction

Human polymorphonuclear leukocytes (neutrophils or PMNs) are mobilized readily to sites of infection and injury where they destroy invading microorganisms and remove damaged tissue and debris (1). PMNs possess a multicomponent NADPH-dependent oxidase which generates superoxide (O_2^-) (for a review see reference 2) and other reactive oxygen species (e.g., H_2O_2, OH•, and HOCl) that contribute greatly to the ability of PMNs to kill invading pathogens (3, 4). In the absence of a functioning oxidase, as is seen in patients with chronic granulomatous disease (CGD)[1] (5), individuals have increased susceptibility to life-threatening bacterial and fungal infections (6).

In resting PMNs, the inactive oxidase is unassembled, with required components segregated into plasma membrane and cytosolic locations (7–9). During activation, required cytosolic components p47-*phox* (10–13), p67-*phox* (10, 14), and Rac2 (15, 16) translocate to the plasma membrane to associate with flavocytochrome b_{558} (7, 17, 18), the key membrane-bound component, thereby assembling the active O_2^--generating complex. Rap1A, a low molecular weight GTP-binding protein associated with flavocytochrome b_{558} (19), and p40-*phox*, another cytosol-derived component (20), appear to have important but as of yet undefined roles in NADPH activation and O_2^--generation.

Flavocytochrome b_{558} appears to contain all of the redox components necessary for the transfer of electrons from NADPH to molecular oxygen, producing O_2^- (21, 22). In resting cells, $\sim$ 10% of flavocytochrome b_{558} is contained within the plasma membrane, 5–15% in the secretory vesicles, and the remainder (75–90%) stored in the membrane of specific granules (23, 24). Flavocytochrome b_{558} is recruited to the plasma membrane from cytosolic granules after stimulation, a process which upregulates the oxidase and enables PMNs to direct production of O_2^- to engulfed pathogens (23–25).

Lipopolysaccharide (LPS or endotoxin) elicits a variety of PMN responses, including receptor upregulation (26, 27), actin assembly (28), and adherence (29), and primes the cell for enhanced release of superoxide (O_2^-) in response to other stimuli such as *N*-formylated bacterial peptides (26, 30–34). Thus, LPS released from invading pathogens at sites of infection may, in a sense, sensitize local phagocytic cells to be more responsive and thereby amplify the inflammatory response. This amplified response may in turn rapidly eliminate the pathogens and/or result in untoward sequelae, such as manifestations of the systemic inflammatory response syndrome. However, LPS itself does not elicit significant O_2^- generation, and the molecular basis of PMN priming by LPS is unknown.

To define further the mechanisms of priming for an enhanced respiratory burst, we investigated the effects of LPS on

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1. *Abbreviations used in this paper:* CE, cell equivalents; CGD; chronic granulomatous disease; DiC8, diacylglycerol; fMLP, *N*-formyl methionyl leucyl phenylalanine; NEPHGE, nonequilibrium pH-gradient electrophoresis.

the NADPH oxidase. Our data indicate that LPS treatment (*a*) upregulated plasma membrane association of flavocytochrome b_{558} and (*b*) significantly enhanced assembly of the cytosolic oxidase factors with the plasma membrane–bound NADPH oxidase after stimulation with *N*-formyl methionyl leucyl phenylalanine (fMLP). The accentuated assembly of the oxidase at the plasma membrane correlated directly with the enhanced respiratory burst observed in intact cells. Our results suggest that increased plasma membrane association of NADPH oxidase components after LPS exposure is the basis for the enhanced respiratory burst observed in LPS-treated PMNs.

Methods

Materials. Endotoxin-free dextran was purchased from USB Biologicals (Cleveland, OH). GTP-γ-*S* and superoxide dismutase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). RNase-free Percoll was purchased from Pharmacia Biotech (Piscataway, NJ) and was assayed for endotoxin content. Endotoxin-free Hypaque (50% diatrizoate sodium injection grade, USP) was obtained from Nycomed (New York, NY). Endotoxin-free 0.9% NaCl and dH_2O (both injection grade, USP) were obtained from Baxter Healthcare Corp. (Deerfield, IL). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless specified.

Endotoxin preparation. LPS was purified from *Salmonella minnesota* as described previously (35). Stock solutions were prepared in Dulbecco's PBS without calcium or magnesium (DPBS) or H_2O and were sonicated 15–30 min (47 kHz at 25°C) in a water bath sonicator (model 1200; Branson Ultrasonics Corp., Danbury, CT) before each use.

Neutrophil isolation. Heparinized, venous blood was obtained from healthy individuals (or from a patient with X-linked CGD) in accordance with a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa, and PMNs were isolated as described previously using Hypaque-Ficoll density-gradient separation after dextran sedimentation (36). Purified PMNs (> 96% of the cells in the preparation) were resuspended in DPBS (or in phosphate-free loading buffer for ^{32}P labeling) and kept on ice until use. All reagents and materials used in the preparation of PMNs (e.g., DPBS, Hypaque-Ficoll, dextran/NaCl, dH_2O, and saline solutions) were essentially endotoxin-free (i.e., < 10.0 pg/ml) as determined by *Limulus* amebocyte lysate assay (QCL-1000; BioWhittaker, Inc., Walkersville, MD).

Neutrophil priming. PMNs ($2–10 \times 10^6$ cells/ml) in DPBS (or loading buffer) were mixed gently at 37°C for the indicated times with LPS (15 or 100 ng/ml, as specified) and then centrifuged at 500 *g* for 10 min at 4°C. Treated PMNs were resuspended in an appropriate assay buffer (either DPBS plus glucose [$DPBS^{++}$/g] for superoxide assays, DPBS for flow cytometry, or relaxation buffer [23] for nitrogen cavitation) at specified cell densities (see below). Untreated PMNs were incubated in a manner identical to that for the LPS-treated cells but without LPS. For fMLP-activated PMNs, cells were incubated with or without LPS, as above, and then with or without 1 μM fMLP at 37°C for the indicated time(s). PMN treatments were terminated by placing cells immediately on ice, and all subsequent procedures were carried out at 0–4°C.

Intact PMN superoxide assays. PMNs were adjusted to 10^7 cells/ml and treated with 15 or 100 ng/ml LPS for 60 min at 37°C. After incubation, LPS-treated or untreated PMNs were incubated with or without 1 μM fMLP at 37°C for an additional 10 min, and O_2^- generation was measured by the superoxide dismutase–inhibitable reduction of ferricytochrome c at 550 nm as described previously (37).

Neutrophil fractionation. PMNs (5×10^6 cells/ml in DPBS) treated with or without LPS for 70 min, or with or without LPS for 60 min then with or without 1 μM fMLP at 37°C for 10 additional min, were treated with 2–4 mM diisopropyl fluorophosphate for 15 min on ice, and then PMN subcellular fractions (plasma membrane– and specific granule–enriched fractions and a cytosol-derived fraction) were isolated using Percoll step gradients as described by Borregaard et al. (23). Plasma membrane– or specific granule–enriched fractions were resuspended in broken-cell assay buffer (38) to 10^9 cell equivalents (CE)/ml for broken-cell superoxide assays or in relaxation buffer (23) for analysis by SDS-PAGE. Cavitates used for quantitative comparison were obtained from an equal number of PMNs.

Broken-cell NADPH oxidase reconstitution assay. For broken-cell assays using SDS as the activating agent, 3×10^6 CE neutrophil cytosol and 6×10^6 CE neutrophil membranes were combined with 100 μM ferricytochrome c, 10 μM FAD, 10 μM GTP-γ-*S*, 100 μM SDS, and buffer (38) to 600 μl final volume. After a 3-min incubation with SDS, NADPH was added to a final concentration of 200 μM, and superoxide dismutase–inhibitable O_2^- production was monitored continuously at 550 nm on a single beam spectrophotometer (model DU 640; Beckman Instruments, Inc., Fullerton, CA) using an Auto 6 sampler (Beckman Instruments, Inc.) for 8–10 min. The concentrations of cytosol and membrane fractions used in these assays were adjusted so that O_2^- generation was not saturating. All samples used for comparison were analyzed simultaneously using the Auto 6 sampler.

Broken-cell assays activated with phosphatidic acid (PA) and diacylglycerol (DiC8) were done essentially as described by McPhail and colleagues (39). The contents of the PA and DiC8 assays were identical to the SDS assays except that 40 μg PA and 10 μg DiC8 replaced SDS, and each assay was incubated at room temperature for 30 min before addition of NADPH.

Spectral analysis of flavocytochrome b_{558}. Plasma membrane– and specific granule–enriched fractions were isolated from untreated and LPS-treated PMNs as described above. The γ-peak at 427 nm in oxidized minus dithionite-reduced difference spectra was used to calculate the amount of flavocytochrome b_{558} in the plasma membrane– or specific granule–enriched fractions as described previously using an extinction coefficient of 106 mM^{-1} cm^{-1} (40). The recovery of flavocytochrome b_{558} from the gradients was 72.2–91.6% as determined by spectroscopy and 87.2–101.7% by SDS-PAGE and subsequent densitometry.

Flow-cytometric analysis of plasma membrane–associated flavocytochrome b_{558}. Plasma membrane association of flavocytochrome b_{558} was determined using an mAb (7D5) which recognizes an extracellular epitope of flavocytochrome b_{558} (41, 42). PMNs (2×10^6 cells/ml in DPBS) treated with or without LPS (100 ng/ml for 5, 10, 20, 40, 60, and 90 min at 37°C) and with or without fMLP (1 μM added to identical untreated and LPS-treated PMNs after incubation for 60 min and further incubated for 1, 2, 5, 10, and 30 min at 37°C) were resuspended in FACS® buffer (DPBS/2% normal goat serum) to a final concentration of 10^7 cells/ml. Plasma membrane association of flavocytochrome b_{558} was detected using 7D5 and a fluorescein-conjugated goat anti–mouse IgG antibody (Organon Teknika Corp., Durham, NC) by the method of Jones et al. (43). After staining, samples were analyzed on a FACScan® flow cytometer (Becton Dickinson, San Jose, CA) at the University of Iowa Core Flow Cytometry facility. Unfixed PMNs were used for all flow-cytometric analyses, and a single live gate was used to eliminate debris and any contaminating cells.

Translocation of NADPH oxidase components and immunoblotting. Plasma membrane–enriched fractions were prepared as described above using the method of Borregaard (23). $1–2 \times 10^7$ CE plasma membrane vesicles obtained from PMNs treated with or without LPS (100 ng LPS/ml for 5, 10, 20, 40, 60, or 70 min as indicated at 37°C) and/or with or without fMLP (1 μM for an additional 10 min at 37°C) were separated using 10% SDS-PAGE and then transferred to nitrocellulose as described previously (see p. 10.2.1 in reference 44). Immunoblots were processed using polyclonal antibody to p47-*phox*, p67-*phox*, and Rac2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by detection using enhanced chemiluminescence (Super Signal substrate; Pierce Chemical Co., Rockford, IL). Plasma membrane association of proteins was quantitated using a scanning laser densitometer (model CS-9000U; Shimadzu Corp., Kyoto, Japan).

Phosphorylation and immunoprecipitation of p47-phox. 1.2×10^8 PMNs were resuspended in 1 ml loading buffer (10 mM Na-Hepes, 138 mM NaCl, 2.7 mM KCl, and 7.5 mM D-glucose, pH 7.5, prepared with endotoxin- and RNase-free reagents: contaminating LPS was removed by filtration through a 1-ml Detoxi-Gel column [Pierce Chemical Co., Rockford, IL]) with 0.5 mCi/ml [^{32}P]orthophosphate and incubated at room temperature for 60 min. After loading, 2×10^7 PMNs were treated with or without LPS (100 ng/ml for 60 min at 37°C) and then with or without fMLP (1 μM for 10 min at 37°C) and iced immediately. After diisopropyl fluorophosphate treatment as above, cells were resuspended in 100 μl lysis buffer (1% Triton X-100, 0.5 mg/ml leupeptin and pepstatin A, 1 mM PMSF, 1 mM sodium orthovanadate, and 0.5% cetyl trimethyl ammonium bromide in Tris-buffered saline, pH 7.5) for 20 min on ice. Insoluble material was removed by centrifugation (14,000 *g* for 30 s at room temperature), and the supernatants were diluted to 1 ml with 50 mM Tris-HCl, 190 mM NaCl containing 2.5% Triton X-100, and 1 mM sodium orthovanadate. P47-*phox* was immunoprecipitated using polyclonal antibody to p47-*phox* and *Staphylococcus* protein A (Pansorbin cells; Calbiochem Corp., La Jolla, CA) using a previously described method (45). *Staphylococcus* protein A–immune complexes were boiled in SDS-sample buffer without reducing agent and resolved with 10% SDS-PAGE. Dried gels were subjected to autoradiography using XAR films (Eastman Kodak Co., Rochester, NY), and the [^{32}P]orthophosphate incorporated into p47-*phox* was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Since the amount of p47-*phox* immunoprecipitated from cells treated with or without LPS and/or fMLP and PMA was compared, we verified that the rabbit antiserum against p47-*phox* immunoprecipitated both native and phosphorylated p47-*phox* with equal efficiency (data not shown). Briefly, 10^7 PMNs were warmed to 37°C and stimulated with buffer alone (control) or 2 μg/ml PMA for 10 min. Cells were lysed and processed as described above for immunoprecipitation. Immunoprecipitates were resolved by 10% SDS-PAGE, and the amount of p47-*phox* was quantitated. The same amount of p47-*phox* was recovered from control or PMA-stimulated cells.

Analysis of p47-*phox* phosphorylation by nonequilibrium pH-gradient electrophoresis (NEPHGE) and SDS-PAGE was performed as described previously (46). Briefly, PMNs treated with or without LPS (100 ng/ml for 60 min at 37°C) and then with or without fMLP (1 μM for 10 min at 37°C) were disrupted by sonication, and the plasma membrane–enriched fraction was isolated as described previously (8). After pH resolution by NEPHGE, gels were subjected to SDS-PAGE and transferred to nitrocellulose. Immunoblots were processed using polyclonal antibody to p47-*phox* and developed using enhanced chemiluminescence (Super Signal substrate).

Results and Discussion

Priming of intact PMNs by LPS. To determine the effect of LPS on PMN O_2^- generation, cells were incubated for 60 min at 37°C with or without LPS, and then subsequently stimulated with 1 μM fMLP for an additional 10 min (Fig. 1). PMNs treated with LPS alone generated very little O_2^- (2.5±0.4 nmole O_2^-/10^6 PMNs vs. 1.4±0.9 for resting cells) (Fig. 1, *bar 2* vs. *bar 1*). However, PMNs treated with LPS produced nearly 10-fold more O_2^- when stimulated subsequently with fMLP (24±1.8 nmole O_2^-/10^6 PMNs) than cells not treated with LPS before fMLP exposure (3.3±0.3) (Fig. 1, *bar 4* vs. *bar 3*). Optimal priming effects in the absence of serum, as a source of LPS-binding protein (47), required pretreatment of PMNs with 100 ng LPS/ml for ~ 60 min as reported previously (30, 34), or as little as 1 ng LPS/ml in the presence of 100 ng LBP/ml (data not shown). Pretreating PMNs with LPS increased greatly the amplitude of the respiratory burst induced by fMLP but did not alter the duration of NADPH oxidase activity (data not shown). The synergy between LPS and fMLP as agonist for PMN O_2^- generation is consistent with previous reports (30, 32, 34, 48).

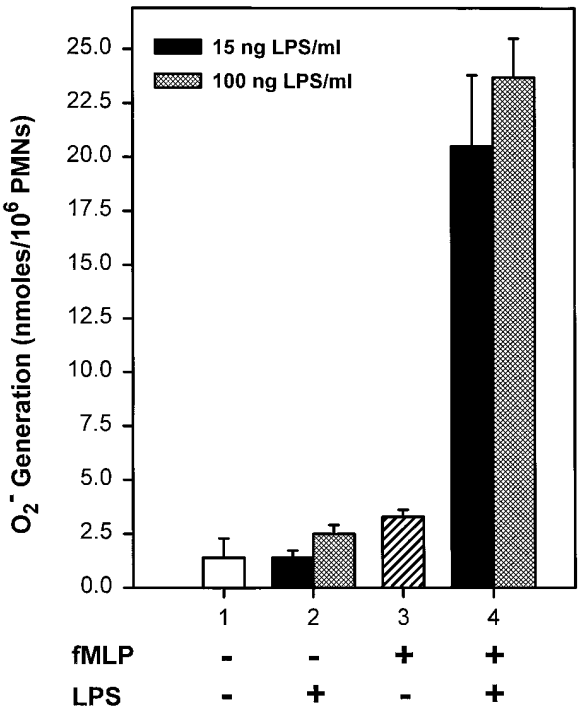

Figure 1. O_2^- generation from PMNs after exposure to bacterial LPS. 5×10^5 PMNs were incubated for 60 min at 37°C with or without LPS (100 ng/ml) and then with or without fMLP (1 μM) for 10 min at 37°C as indicated. O_2^- generation was determined as described in Methods, and results are expressed as the mean±SD of four separate experiments.

Enhanced O_2^- production by plasma membrane–rich fractions of LPS-primed PMNs. Because the NADPH oxidase catalyzes the production of O_2^-, we used a broken-cell NADPH oxidase reconstitution system to test the hypothesis that LPS directly affects NADPH oxidase components. PMNs treated with or without LPS were fractionated, and the O_2^--generating activities of the plasma membrane– and specific granule–enriched fractions were compared. As shown in Fig. 2, incubation of cytosol and plasma membrane–rich fractions from LPS-treated PMNs produced approximately two times more O_2^- (1.2±0.24 nmole O_2^-/min/10^7 CE; *bar 3*) than analogous fractions from untreated cells (0.63±0.28; *bar 1*) in the SDS-activated broken-cell assay. Similar results were obtained in a broken-cell system activated by PA and DiC8, agonists which might better represent signaling pathways triggered by fMLP (39, 49). Again, cytosol and plasma membrane–rich fractions from LPS-treated PMN elicited nearly twofold greater O_2^--generating activity (0.49±0.24 nmole O_2^-/min/10^7 CE) compared with cytosol and plasma membrane–rich fractions from untreated cells (0.26±0.14) (Fig. 2, *bar 6* vs. *bar 4*). A similar enhancement of broken-cell O_2^--generating activity was observed when plasma membrane–rich fractions (from LPS-pretreated PMNs) were combined with cytosol obtained from either untreated or LPS-treated cells (compare *bars 2* and *3* and *bars 5* and *6* in Fig. 2). Thus, most of the enhanced

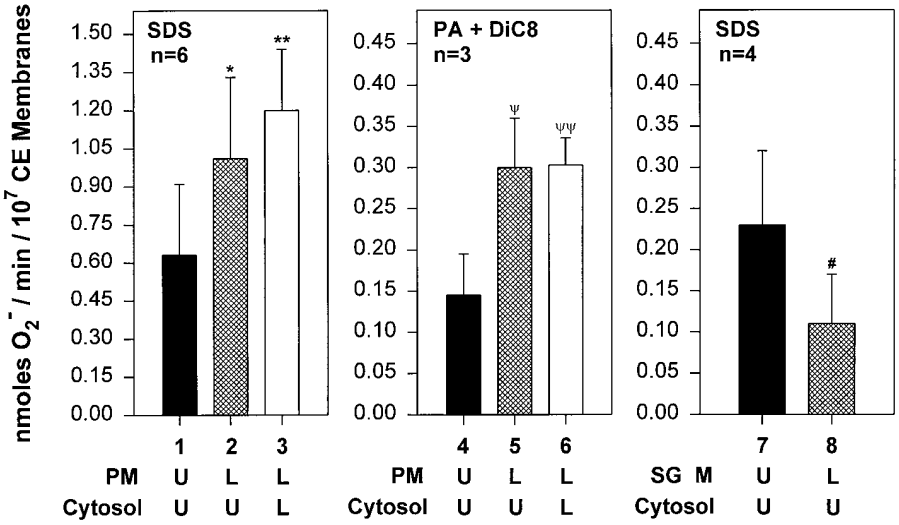

Figure 2. O_2^--generating activity in plasma membrane– and specific granule–enriched fractions from LPS-treated PMNs. PMNs were treated with or without LPS (*U*, untreated cells; *L*, LPS-treated cells) and then fractionated for use in broken-cell assays as described in Methods. Plasma membrane–rich fractions (*PM*) and cytosol from untreated or LPS-treated PMNs were combined as indicated and then assayed for O_2^--generating activity using SDS (*bars 1–3*) or PA + DiC8 (*bars 4–6*) as agonists. Specific granule–rich fractions (*SG M*) from untreated (*bar 7*) or LPS-treated PMNs (*bar 8*) were combined with cytosol from untreated PMNs, and O_2^- generation was determined as described in Methods. Results are expressed as the mean±SD of three to six separate experiments. The significance of O_2^- generation versus that of plasma membrane–enriched and cytosolic fractions from untreated PMNs was determined by paired Student's *t* test: $^*P = 0.0047$, $^{**}P = 0.0042$, $^{\Psi}P = 0.02$, $^{\Psi\Psi}P = 0.00062$, $^{\#}P = 0.01$.

broken-cell O_2^--generating activity reflected changes in the properties of the plasma membrane–rich fractions derived from LPS-treated PMNs.

LPS induces mobilization of flavocytochrome b_{558} to the plasma membrane. It should be noted that whereas plasma membrane–rich fractions from LPS-treated cells showed increased NADPH oxidase activity when incubated with cytosol plus SDS or PA plus DiC8, these membrane fractions in the absence of added cytosol expressed very little activity (0.25±0.03 nmole O_2^-/min/10^7 CE for assays without added cytosol vs. 1.2±0.24 for assays containing added cytosol); O_2^--generating activity of plasma membrane–rich fractions (without added cytosol) from resting cells is 0.13±0.02 nmole O_2^-/min/10^7 CE). These findings seem most compatible with a mobilization of flavocytochrome b_{558}–containing specific granules to the plasma membrane during PMN treatment with LPS rather than preassembly of the active O_2^--generating complex. To test more directly the effect of LPS treatment on the subcellular localization of flavocytochrome b_{558}, we quantitated flavocytochrome b_{558} in plasma membrane–rich fractions and in specific granules isolated from control and LPS-treated PMNs using oxidation-reduction difference spectroscopy. Spectral analysis revealed an increase in flavocytochrome b_{558} in plasma membrane–rich fractions and a corresponding decrease in flavocytochrome b_{558} from the specific granule–rich fractions after LPS treatment (Table I), consistent with an induction of specific granule exocytosis by LPS. The changes in flavocytochrome b_{558} content of plasma membrane–rich fractions and specific granules paralleled changes in broken-cell NADPH oxidase activity expressed by these fractions in concert with cytosol. Thus, whereas the activity of plasma membrane–rich fractions (Fig. 2, *bars 1–6*) was increased by LPS treatment, the activity of the specific granules was decreased (Fig. 2, *bars 7* and *8*).

The data shown in Table I reproduce the subcellular distribution of flavocytochrome b_{558} in control PMNs reported previously (23, 24), and suggest that upregulation of plasma membrane–associated flavocytochrome b_{558} by LPS is the result of granule–plasma membrane fusion. However, one limitation of these data is that secretory vesicles copurify with plasma membrane vesicles using Percoll gradients (25), and, therefore, flavocytochrome b_{558} recovered in plasma membrane–rich fractions of resting cells is derived from both plasma membrane and secretory vesicles. However, it is likely LPS elicits secretory vesicle mobilization before that of specific granules, in accordance with a reported hierarchy of granule exocytosis (25, 50).

To monitor more directly the mobilization of flavocytochrome b_{558} to the plasma membrane during LPS treatment, PMNs were analyzed by flow cytometry using an mAb (7D5) which recognizes an extracytoplasmic epitope of flavocytochrome b_{558}. FACScan analysis indicated 7D5 bound PMNs from healthy individuals, but did not bind to PMNs from a patient with X-linked CGD, indicating the specificity of 7D5 for

Table I. Subcellular Distribution of Flavocytochrome b_{558} after LPS Exposure

PMN treatment	Plasma membrane		Specific granule membrane	
	conc. (pmol)	*% total*	*conc. (pmol)*	*% total*
Untreated PMNs	4.33±0.85	(25.9%)	12.36±5.7	(74.1%)
LPS-treated PMNs	6.42±0.66	(40.6%)*	9.34±4.6	(59.1%)‡

PMNs incubated with or without LPS were fractionated, and flavocytochrome b_{558} in plasma membrane– and specific granule–enriched fractions was quantitated by oxidation-reduction spectroscopy. Flavocytochrome b_{558} concentration (from 10^7 CE) is expressed as the mean±SD of four separate experiments. Statistical analyses were performed using the paired Student's *t* test. $^*P = 0.007$ vs. untreated plasma membranes. $^‡P < 0.05$ vs. untreated specific granule membranes.

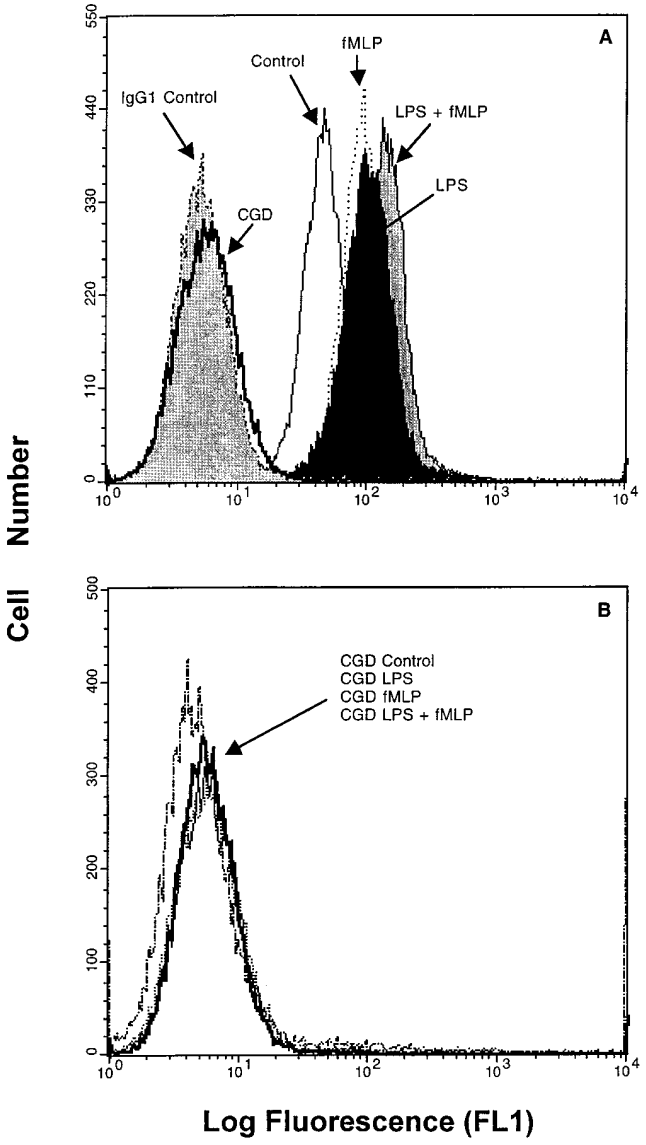

Figure 3. Plasma membrane association of flavocytochrome b_{558} after LPS treatment. PMNs from healthy subjects (*A*) or from an individual with X-linked CGD (*B*) were treated with or without LPS (60 min at 37°C with 100 ng LPS/ml) and subsequently with or without fMLP (1 μM) for an additional 10 min and then probed with 7D5, an mAb which recognizes an extracytoplasmic epitope of flavocytochrome b_{558}. PMNs stained with a control IgG1 mAb of the same isotype as 7D5 and untreated PMNs from an individual with CGD were included for comparison (*A*). Results are from one experiment representative of five (*A*, healthy cells) or two (*B*, CGD cells).

flavocytochrome b_{558} (Fig. 3 *A*). Furthermore, PMNs treated with or without LPS (100 ng/ml for up to 90 min at 37°C) and subsequently with or without fMLP (1 μM for 10 min at 37°C) revealed a hierarchy of stimuli for increasing plasma membrane association of flavocytochrome b_{558}: LPS + fMLP > fMLP ≥ LPS > control PMNs. In contrast, PMNs from a patient with X-linked CGD did not show increased plasma membrane–associated flavocytochrome b_{558} in response to LPS, fMLP, or to a sequential treatment of LPS and fMLP (Fig. 3 *B*). Although addition of fMLP to LPS-pretreated PMNs further augmented the translocation of flavocytochrome b_{558} to the plasma membrane, the increase in cell surface flavocytochrome b_{558} at the time of maximal activation of the NADPH oxidase activity by fMLP (< 2 min [51]) was very limited (Fig. 4). Thus, it is unlikely that this effect was entirely responsible for the dramatic increase in NADPH oxidase activity observed promptly after fMLP addition to LPS-primed cells. In contrast, the time-dependent mobilization of flavocytochrome b_{558} to the plasma membrane induced by LPS closely paralleled LPS priming (references 30, 32, 34, and 52, and data not shown), suggesting that LPS-induced translocation of flavocytochrome b_{558} may underlie LPS priming of the fMLP-triggered respiratory burst.

Translocation of p47/67-phox and Rac2 from cytosol to the plasma membrane occurs mainly after addition of fMLP to LPS-primed PMNs. The absence of significant NADPH oxidase activity either in intact cells or plasma membrane–enriched, cytosol-free fractions after LPS treatment despite the mobilization of flavocytochrome b_{558} suggests that essential cytosol-derived cofactors of the oxidase may not be mobilized during LPS treatment. To test this hypothesis, plasma membrane–enriched fractions from PMNs treated with or without LPS and subsequently with or without fMLP were isolated and probed for associated p47-*phox*, p67-*phox*, and Rac2. Plasma membrane–rich fractions from PMNs treated with LPS alone showed a small (approximately threefold) increase in p47-*phox* but little increase in either p67-*phox* or Rac2 (Fig. 5, *A* and *B*). Time-course analysis of LPS-induced mobilization of p47-*phox* to the plasma membrane–enriched fraction indicated

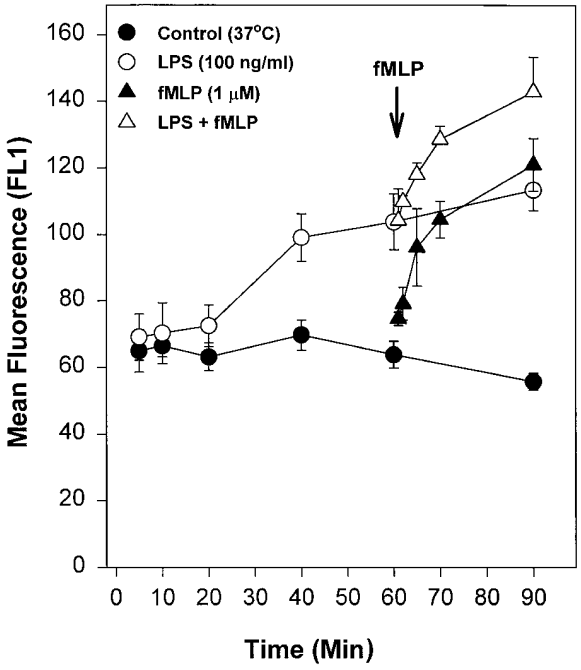

Figure 4. Time-dependent mobilization of flavocytochrome b_{558} to the plasma membrane after LPS treatment. PMNs from healthy subjects were treated with (○, △) or without (●, ▲) 100 ng LPS/ml for the indicated times at 37°C and probed with 7D5, or were stimulated subsequently with fMLP (1 μM) (△, ▲) for the additional indicated times and then probed with 7D5. Results are expressed as the mean fluorescence±SD of three to five separate experiments.

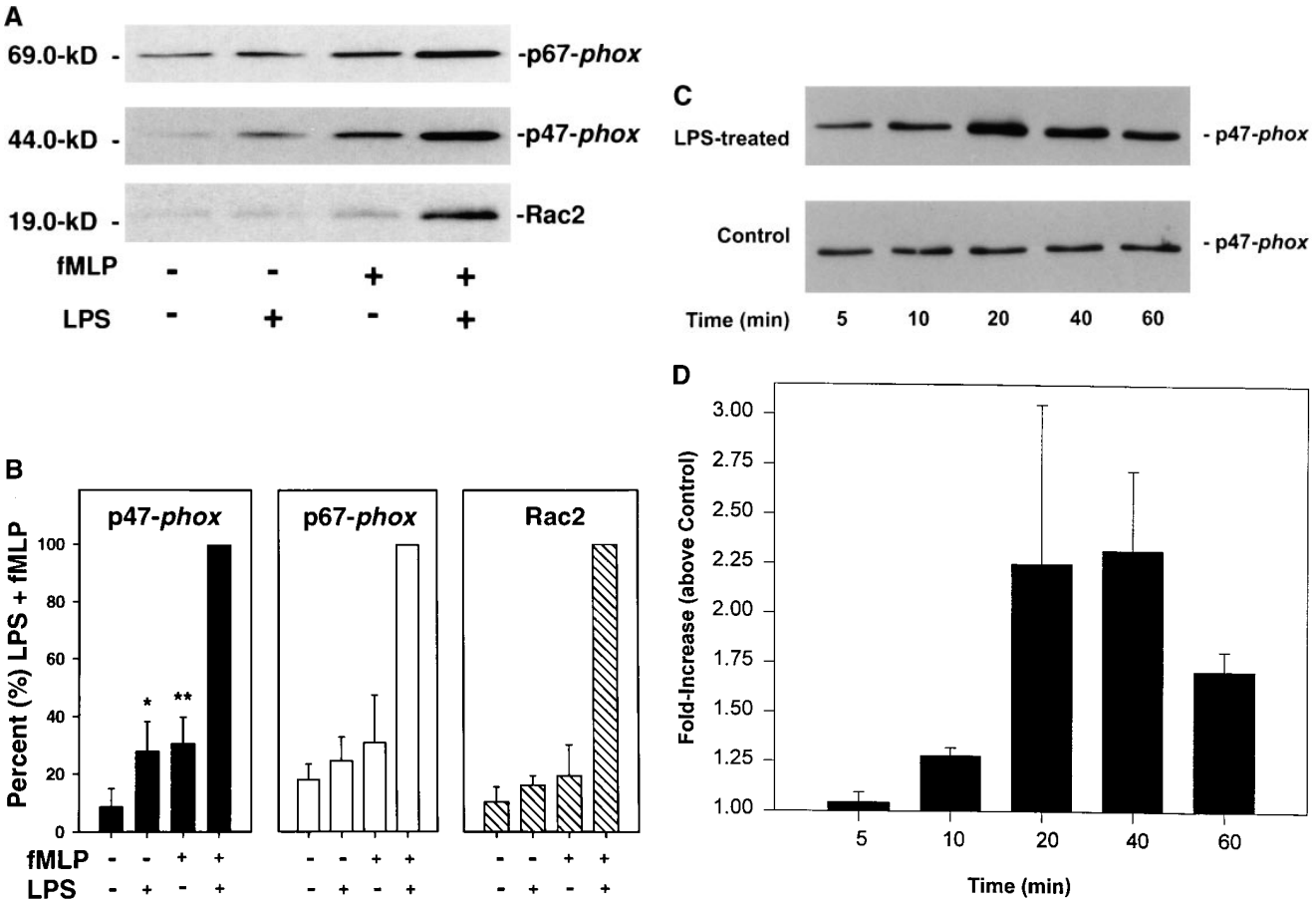

Figure 5. Analysis of plasma membrane–associated p47/67-*phox* and Rac2 in PMNs after exposure to bacterial LPS. Plasma membrane–rich fractions isolated from PMNs treated with or without LPS (60 min at 37°C with 100 ng LPS/ml) and subsequently with or without fMLP (10 min at 37°C with 1 μM fMLP) as indicated were probed for associated p47-*phox*, p67-*phox*, and Rac2 (*A*) and then quantitated by densitometry (*B*). Alternatively, plasma membrane–rich fractions isolated from PMNs treated with or without 100 ng LPS/ml for the indicated times were probed for associated p47-*phox* (*C*) and then quantitated by densitometry (*D*). Results in *A* are from one experiment representative of three. Translocation is expressed as a percentage of the fully activated assay (LPS-treated followed by fMLP-stimulated; % *LPS + fMLP*), and is the mean±SD of at least three separate experiments (*B*). Results in *C* are from one experiment representative of two. These results are also represented densitometrically in *D*, and are expressed as the mean±SD of two separate experiments.

that translocation had similar kinetics as the LPS-induced upregulation of flavocytochrome b and that of LPS priming in general (30, 33, 34), providing additional evidence that LPS priming for an augmented respiratory burst is the direct result of redistribution of NADPH oxidase components (Fig. 5, *C* and *D*). Analogous fractions from PMNs treated with fMLP alone showed a similarly small increase in all three cytosolic oxidase components (Fig. 5, *A* and *B*). However, when fMLP was added to LPS-pretreated PMNs, translocation of all three cytosolic protein species was enhanced dramatically; the levels of p47-*phox*, p67-*phox*, and Rac2 in the plasma membrane–enriched fractions were ~ 10-fold above normal resting levels (Fig. 5, *A* and *B*). The combined effect of LPS and fMLP on the translocation of p47-*phox*, p67-*phox*, and Rac2 is much greater than additive, and correlates well with the relative levels of O_2^--generating activity expressed by intact PMNs under these different conditions.

Phosphorylation of p47-phox induced by LPS and/or fMLP. Previous studies have shown that phosphorylation and translocation of p47-*phox* correlate with activation of the respiratory burst in PMNs (46, 53, 54). To investigate further the possible role of p47-*phox* phosphorylation in LPS priming, we analyzed the effects of LPS pretreatment on the phosphorylation of p47-*phox* in fMLP-stimulated PMNs. LPS pretreatment of PMNs caused a detectable, albeit slight increase in the phosphorylation of p47-*phox* versus control cells (Fig. 6 *A*). Phosphorylation of p47-*phox* was increased roughly fivefold when PMNs were treated with fMLP alone but was enhanced only slightly further in cells pretreated with LPS before addition of fMLP (Fig. 6 *A*). Thus, whereas translocation of p47-*phox* correlated with augmented O_2^--generating activity in cells treated sequentially with LPS and fMLP (compare Figs. 1 and 5), phosphorylation of p47-*phox* after fMLP stimulation did not appear similarly enhanced by LPS pretreatment (compare Figs. 1 and 5 with Fig. 6). Because the results shown in Fig. 6 *A* include combined membrane and cytosolic pools of p47-*phox*, it could be argued that differences in the plasma membrane–bound phosphorylated p47-*phox* might be masked by additional phosphorylated cytosolic species. Plasma membrane–rich fractions from PMNs pretreated with LPS and then

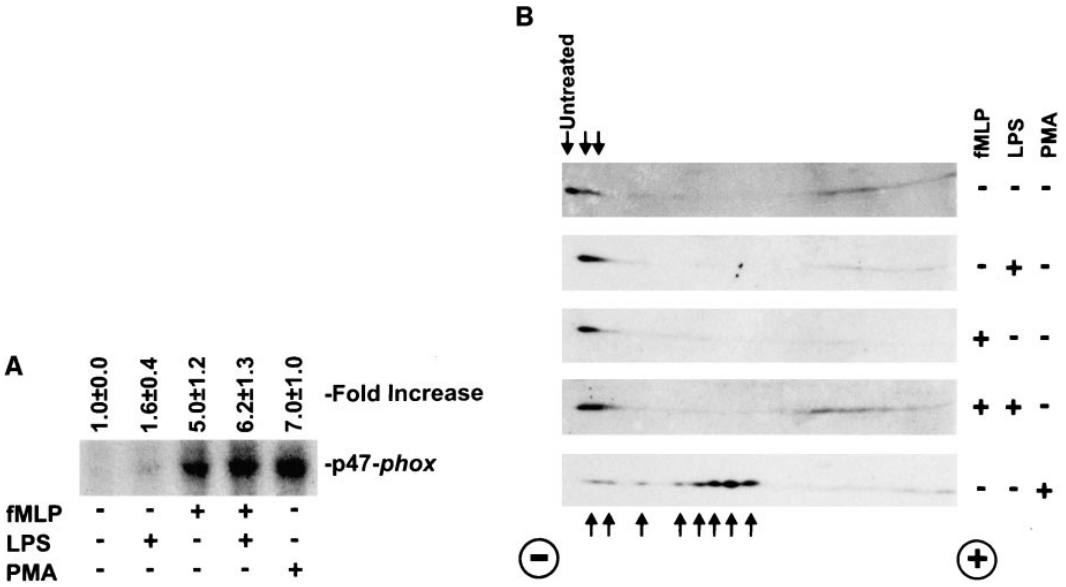

Figure 6. Effect of LPS treatment on the phosphorylation of p47-*phox*. PMNs loaded with [^{32}P]orthophosphate were treated with or without LPS (60 min at 37°C with 100 ng LPS/ml) and subsequently with or without fMLP (1 μM fMLP for 10 min at 37°C) as indicated, and p47-*phox* was immunoprecipitated as described in Methods (*A*). The immunoprecipitate from PMNs stimulated with 2 μg/ml PMA was included for comparison (*A*). Alternatively, plasma membrane–enriched fractions from PMNs treated with or without LPS (60 min at 37°C with 100 ng LPS/ml) and subsequently with or without fMLP (1 μM fMLP for 10 min at 37°C) as indicated were analyzed by two-dimensional gel electrophoresis as described in Methods (*B*). *B* is an immunoblot probed with p47-*phox* antibody. Since there was less immunoreactive p47-*phox* in plasma membrane–rich fractions of untreated PMNs (*top*), it was necessary to expose that immunoblot longer to x-ray film in order to visualize a signal which would serve as a comparative standard for immunoblots of analogous fractions after specified treatments. *Arrows*, Relative position of various p47-*phox* species; electrode charge during NEPHGE is indicated (*bottom*). Results are from one experiment representative of two, and quantitation is expressed as fold-increase relative to phosphorylation in untreated PMN immunoprecipitates (1.0), and is the mean±SD of two separate experiments.

treated with fMLP do not show a substantially greater accumulation of more acidic (i.e., phosphorylated) p47-*phox* species than do cells at rest or after treatment with LPS or fMLP alone (Fig. 6 *B*). The limited effects of LPS and/or fMLP on phosphorylation of p47-*phox* are particularly striking when juxtaposed with the effects of PMA, both quantitatively and qualitatively. Incorporation of ^{32}P into p47-*phox* was nearly sevenfold greater after PMA treatment than after LPS alone (Fig. 6 *A*), whereas incorporation of ^{32}P into p47-*phox* after a sequential treatment of LPS and fMLP was similar in magnitude to that of PMA (Fig. 6 *A*). By contrast, plasma membrane–rich fractions from PMNs treated with PMA contained at least eight acidic derivatives of p47-*phox*, including several species more acidic than the most anodal species accumulating after treatment with LPS and/or fMLP. Analogous fractions obtained from PMNs treated with LPS, fMLP, or LPS and fMLP sequentially contained only one or two more acidic species of p47-*phox* than did untreated PMNs (Fig. 6 *B*). These findings suggest that even limited phosphorylation of p47-*phox* induced by LPS and/or fMLP may play an important role in p47-*phox* translocation and respiratory burst activation (46, 53–55). Since there are few anodal plasma membrane–associated species of p47-*phox* in any of the LPS/fMLP combinations (Fig. 6 *B*), the effects of LPS and fMLP may only transiently involve phosphorylation of p47-*phox* or be limited to one or two important phosphorylation sites affecting a small percentage of total p47-*phox.* However, we cannot exclude the possible existence of an alternative phosphorylation-independent pathway of NADPH oxidase activation as suggested previously by Robinson et al. (56), or a possible lack of correlation between phosphorylation and translocation with certain agonists as reported previously (51). Studies are currently under way to determine the location and kinetics of these limited LPS- and/or fMLP-induced phosphorylation events.

In summary, our data suggest that LPS priming for enhanced O_2^- generation by fMLP-stimulated PMNs reflects redistribution of NADPH oxidase components, thereby facilitating increased assembly of the NADPH oxidase. The effects of LPS and fMLP on this assembly process appear to be distinct: LPS pretreatment induces mainly translocation of flavocytochrome b_{558} from specific granules to the plasma membrane, with limited translocation of p47-*phox* but little concomitant translocation of p67-*phox* or Rac2 from the cytosol. In contrast, subsequent treatment with fMLP rapidly induces translocation of all three cytosolic components to the plasma membrane, with little additional recruitment of flavocytochrome b_{558}. Previous attempts (30, 32) antedated the more detailed characterization of cytosolic components and precise definition of the subcellular distribution of the flavocytochrome b available today. Specifically, the method used by Forehand et al. to isolate plasma membrane vesicles also coisolated specific granules (32). Therefore, LPS-augmented, plasma membrane–associated O_2^--generating activity resulting from flavocytochrome b redistribution would have gone unnoticed as reported (32).

The events reported here appear to be directly responsible for the enhanced respiratory burst observed in LPS-primed PMNs, since translocation of cytosolic oxidase components is closely associated with O_2^- generation (8, 9). However, it is apparent that other more proximal events result in upregulation of the NADPH oxidase after LPS exposure and involve a variety of signaling pathways (28, 32, 33, 52). Recently, El Benna

and co-workers proposed that p47-*phox* could be phosphorylated by p38 mitogen-activated protein kinase (57), a kinase reported by Nick et al. to be activated by LPS (28). Thus, the importance of LPS-induced phosphorylation pathways on NADPH oxidase activation remains to be determined.

In view of the acute but short-lived activation of the respiratory burst that is triggered by fMLP (30, 49), we propose that LPS priming of this process represents cellular and/or biochemical alterations that increase the efficiency of NADPH oxidase assembly and subsequent generation of O_2^-. The increased efficiency with which O_2^- can be generated translates to more efficient microbial killing and an overall better host response to invading microorganisms. Characterization of the molecular and cellular basis for LPS-dependent mobilization of flavocytochrome b_{558} and modulation of specific signaling events involved in translocation of p47-*phox*, p67-*phox*, and Rac2 awaits further study.

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