PRIMING OF NEUTROPHILS FOR ENHANCED RELEASE OF OXYGEN METABOLITES BY BACTERIAL LIPOPOLYSACCHARIDE

Evidence for Increased Activity of the Superoxide-producing Enzyme

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Mononuclear phagocytes are known to be long-lived cells, capable of synthesis of new cellular constituents and of major alterations in metabolic activities and morphology (1). In contrast, the neutrophil generally has been considered an end-stage cell, incapable of significant functional or metabolic modulation. However, Granelli-Piperno et al. (2, 3) have reported that human neutrophils actively synthesize protein and RNA and secrete plasminogen activator when placed in culture, and that these activities can be stimulated by incubation of the cells with concanavalin A and inhibited by incubation with glucocorticoids. These findings, and the capacity of small amounts of bacterial endotoxin (lipopolysaccharide, LPS)[1] to prime mononuclear phagocytes for enhanced stimulated release of superoxide anion (O_2^-) (4, 5), suggested to us that neutrophil function might be modified by exposure to LPS. We report here that incubation of human neutrophils for 30–60 min with LPS primes these cells for enhanced release of O_2^- or hydrogen peroxide (H_2O_2) upon subsequent contact with a variety of stimuli. This increase in oxidative metabolism might permit increased effectiveness of the neutrophil in host defense. These results also raise the possibility that exposure of neutrophils to bacterial LPS could enhance the capacity of these cells to damage tissue, which might occur in conditions such as endotoxic shock or infection-induced adult respiratory distress syndrome.

Materials and Methods

Preparation of Neutrophils. Human neutrophils were isolated from venous blood anticoagulated with 10% sodium citrate (3.8% solution in water; Fisher Scientific Co., Pittsburgh, PA). 3 vol blood were added to 1 vol 6% dextran (70,000 mol wt) in 0.9% sodium chloride solution (Cutter Laboratories, Berkeley, CA) and allowed to stand at

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; FMLP, formyl-methionyl-leucyl-phenylalanine; KRPD, Krebs-Ringer phosphate buffer with dextrose, 2 mg/ml; LPS, lipopolysaccharide; O_2^-, superoxide anion; PBS, phosphate-buffered saline, pH 7.4; PMA, phorbol myristate acetate; TCA, trichloroacetic acid.

room temperature for 1 h. The leukocyte-rich plasma was aspirated and centrifuged, and the erythrocytes were removed by hypotonic lysis (6). The cells were washed twice and resuspended in Krebs-Ringer phosphate buffer, pH 7.23, with 0.2% dextrose (KRPD), prepared with dextrose in 0.9% NaCl solution (Cutter Laboratories). The final cell preparation contained 80–89% neutrophils. Neutrophils, made >99% pure by the use of Ficoll-Hypaque gradients (6) containing large amounts of LPS or of plasma and Percoll (Sigma Chemical Co., St. Louis, MO) by a modification of a method previously described (7), were capable of being fully primed by incubation with LPS. Values obtained with control cells (no incubation with LPS), however, were elevated compared with results achieved with cells obtained by dextran sedimentation.

All reagents (in concentrated stock solutions) and plastic ware used in the preparation of cells and in assays were tested for LPS contamination by the limulus amebocyte lysate assay (Sigma Chemical Co.) (4) and were determined to be free of detectable LPS. (The limulus assay was capable of detecting 0.1 ng/ml of reagent LPS.) Plastics were tested by placing the limulus solution directly into the plastic ware, incubating at 37°C for 1 h, and examining for gelation of the solution.

Priming of Neutrophils with LPS. Cells were incubated at 37°C for varying periods of time with KRPD containing LPS extracted with phenol from *Escherichia coli* K235 (4, 5, 8) (a gift from Dr. Floyd McIntire, University of Colorado School of Dentistry) or with KRPD alone. This LPS was free of lipid A–associated endotoxic protein and contained no detectable ubiquinone (4, 8). LPS from *E. coli* 0111:B4, 026:B6, 0127:B8, and 055:B5 (List Biological Laboratories, Campbell, CA), *E. coli* 0128:B12 (Sigma Chemical Co.), *Salmonella minnesota* Re 595 (List Biological Laboratories), and *S. abortus equii* (Difco Laboratories, Detroit, MI), and lipid A from *S. minnesota* Re 595 (List Biological Laboratories) also were found to prime neutrophils for enhanced O_2^- release (60-min pretreatment with 10–100 ng/ml LPS at 37°C); however, most of the experiments reported here used LPS from *E. coli* K235. At the end of the pretreatment time, cells were determined to be >98% viable by the exclusion of trypan blue. Neutrophil clumping did not occur, as determined by microscopy of cells in suspension. Stimuli for O_2^- or H_2O_2 release were added without removal of the LPS; in seven paired experiments, the effect of LPS on O_2^- release was almost identical in preparations in which the LPS-containing buffer was removed after 10–60 min of preincubation and the cells were washed and again preincubated at 37°C for a total of 60 min before addition of the stimulus.

Preparation of Stimuli. Complexes of bovine serum albumin (BSA) (Sigma Chemical Co.) and antibody to BSA raised in rabbits were formed at equivalence at 4°C in phosphate-buffered saline, pH 7.4 (PBS) and adhered to micropore filters (25 mm diam, 5 μm pore size; Millipore Corp., Bedford, MA). Filters were washed five times with PBS before addition of cells (9).

Phorbol myristate acetate (PMA) was obtained from Consolidated Midland Corp., Brewster, NY and diluted in dimethylsulfoxide (grade I; Sigma Chemical Co.) to a concentration of 450 ng/ml. Formyl-methionyl-leucyl-phenylalanine (FMLP) was obtained from Vega Biochemicals, Tucson, AZ and diluted in saline to a stock concentration of 0.1 mM.

Release of O_2^- by Neutrophils. Release of O_2^- was determined in duplicate or triplicate reaction mixtures, as previously described (6, 9), by superoxide dismutase–inhibitable reduction of cytochrome *c* (type III; Sigma Chemical Co.). Micropore filters to which preformed immune complexes had been fixed were placed in individual 35-mm diam tissue culture dishes (Falcon Labware, Oxnard, CA). A mixture of cytochrome *c* and KRPD, with or without superoxide dismutase (Diagnostic Data Inc., Berkeley, CA), was added to each dish; and 4×10^6 cells that had been pretreated with LPS or with KRPD alone were added to start the reaction. The mixtures were incubated at 37°C for 60 min, then aspirated and iced immediately. Cells were removed by centrifugation and the A_{550} of the supernatant was determined.

In assays using PMA (1.5 ng/ml to 1 μg/ml) or FMLP (1 μM) as the stimulus, O_2^- was measured in a similar manner, except that the cells were gently agitated at 37°C in a shaking water bath and kept in suspension in polypropylene tubes. The incubation time

was 10 min and 2.5×10^6 neutrophils were used. In both assay systems, the reaction volume was 1.5 ml, the cytochrome *c* concentration was 0.08 mM, and the concentration of superoxide dismutase, when present, was 50 μg/ml.

Release of O_2^- was measured continuously in a Cary 219 double-beam spectrophotometer fitted with heated (37°C) and stirred cuvettes and a recorder. Neutrophils (2.5×10^6), preincubated with LPS, 10 ng/ml, or buffer were added to paired cuvettes containing 0.08 mM cytochrome *c* in KRPD; the reference cuvette also contained superoxide dismutase, 50 μg/ml. The assay volume was 3 ml. After stabilization of the tracing (1–2 min), 1 μM FMLP was added as stimulus. Assays were performed in duplicate or triplicate.

Release of H_2O_2 and Consumption of Oxygen by Neutrophils. Release of H_2O_2 was measured by quantitating peroxidase-mediated oxidation of scopoletin by H_2O_2 using a modification of a previously described technique (9, 10). Horseradish peroxidase, scopoletin (10), and 5×10^6 granulocytes were present in the 3-ml reaction mixture, which was not stirred. The decrease in fluorescence of scopoletin was measured continuously in a recording spectrofluorometer at 37°C after addition of 1 μM FMLP as stimulus. Assays were performed in duplicate and results are reported as initial (maximal) rates.

Consumption of oxygen by neutrophils was measured continuously at 37°C by a polarographic technique using a Clark membrane electrode (Yellow Springs Instrument Co., Yellow Springs, OH). Neutrophils were preincubated with 10 ng/ml LPS or buffer alone at 37°C for 60 min. The 3-ml assay volume contained $2.5–20 \times 10^6$ neutrophils and 2 mM KCN in KRPD. After stabilization of the tracing, 1 μM FMLP was added to start the reaction. Results varied directly with cell number within the range studied ($2.5–20 \times 10^6$). Assays were performed in duplicate or triplicate. Results are expressed as initial (maximal) rates.

Effect of Cycloheximide on Priming of Neutrophils by LPS. Cycloheximide (Sigma Chemical Co.), 1–50 μg/ml, was preincubated with neutrophils at 37°C for 15 min before addition of the LPS for the 60-min preincubation. That cycloheximide inhibited neutrophil protein synthesis in this system was demonstrated by studying incorporation of [^{14}C]leucine. Neutrophils were resuspended in medium 199 without amino acids (Gibco Laboratories, Grand Island, NY) at 1×10^7/ml. [^{14}C]Leucine (0.5 μCi/ml) (New England Nuclear, Boston, MA) was added with or without cycloheximide, with or without 10 nM FMLP. Cells and supernatant were harvested at 2 or 24 h and precipitated with an equal volume of cold 20% trichloracetic acid (TCA) in the presence of added albumin as a carrier. The precipitate was washed and counted for incorporated radioactivity. FMLP stimulated incorporation of [^{14}C]leucine into TCA-precipitable material by approximately twofold. Cycloheximide (10 μg/ml) inhibited the incorporation by >90% in both resting and stimulated conditions, at both 2 and 24 h. Cellular integrity, checked by examination of the release of lactate dehydrogenase (9) in parallel tubes, was equivalent in the presence or absence of cycloheximide.

Binding of FMLP to Neutrophils. FMLP binding to neutrophils was studied by a modification of the technique described for rabbit neutrophils by Mackin et al. (11). Neutrophils at 1×10^7/ml were preincubated in buffer or buffer with LPS, 10 ng/ml, for 60 min at 37°C. 250 μl of this cell suspension was layered onto silicone fluid with an equal volume of F-met-leu-^{3}H phe (New England Nuclear). The tubes were incubated at 4°C for 30 min, and then the cells were separated from unbound peptide by centrifugation for 30 s in a microcentrifuge. The pellets were solubilized and counted. Binding data were analyzed with the LIGAND program (11). Analysis of the Scatchard plots did not reveal evidence for two populations of binding sites on these cells, consistent with the report of Koo et al. (12) for intact human neutrophils. Because of the concerns about Scatchard analysis raised by Munson and Rodbard (13), we examined a wide range of FMLP concentrations in these studies (2.7–32.4 pmol per tube).

Release of O_2^- by Particulate Fractions of Neutrophils. Release of O_2^- was measured in particulate fractions prepared from neutrophils pretreated with 10 ng/ml LPS for 60 min, and from control neutrophils, using a modification of a previously described technique (14). After preincubation with LPS, the cells were treated for 3 min at 37°C with 1 μg/ml PMA, disrupted by sonication, and centrifuged, first at 500 *g*, then at 9,500 *g*.

The supernate was centrifuged again to yield a 40,000 *g* pellet containing the activated enzyme. ~75% of the NADPH-dependent O_2^--producing activity of the crude cell sonicate is present in this pellet (15). The pellet was suspended in 0.34 M sucrose and assayed for superoxide dismutase–inhibitable NADPH-dependent reduction of cytochrome *c*, as previously described (14), except that sodium deoxycholate (Sigma Chemical Co.), 0.042%, was present in the reaction mixture (16). The total amount of protein in the pellet (14) and the activity of the plasma membrane marker alkaline phosphatase (10) were almost identical in particulate fractions made from PMA-stimulated neutrophils preincubated with LPS or with buffer. In one of the experiments plotted in Fig. 3, using an optimal concentration of NADPH (0.19 mM) the enhancement in O_2^--producing activity after pretreatment of the cells with LPS (compared with buffer control) was equivalent in the crude cell sonicate and in the final particulate fraction, indicating that recovery of the oxidase was not affected by exposure of the cells to LPS.

Results

LPS-induced Priming for Enhanced Release of O_2^-

Human neutrophils exposed to small amounts of LPS in vitro could be primed so that they released increased amounts of O_2^- in response to a variety of stimuli. As summarized in Table I, neutrophils pretreated for 60 min with 10 ng/ml LPS, when allowed to contact surface-bound immune complexes or when exposed to the soluble stimuli PMA or FMLP, released ~50% more to almost eight times more O_2^- than did the same preparation of neutrophils preincubated without LPS. In itself, LPS was not a stimulus for O_2^- release, whether O_2^- release was assayed from the time of the addition of LPS or after a 60-min pretreatment period with LPS (Table I).

Neutrophils were capable of being primed by a wide range of concentrations

TABLE I

*Effect of Preincubation of Neutrophils with LPS on the Release of O_2^- During Stimulation**

Stimulus	Release of O_2^-‡ (nmol/2.5 × 10^6 neutrophils)	
	Neutrophils preincubated with:	
	Buffer	LPS
None (16)§	2.0 ± 0.2	2.1 ± 0.1
PMA, 1.5 ng/ml (12)	49.0 ± 5.2	80.6 ± 4.8
Fixed immune complexes (21)	12.4 ± 1.6	26.8 ± 1.6
FMLP, 1 μM (35)	5.0 ± 0.4	38.8 ± 2.2

* Cells were preincubated for 60 min with buffer (KRPD) or with KRPD containing LPS, 10 ng/ml. Stimulation with immune complexes was for 60 min and with PMA or FMLP for 10 min. Incubation in the absence of a stimulus was for 10 min, in suspension.

‡ Mean ± SEM. The number of paired experiments, performed in duplicate or triplicate, is given in parentheses by the stimulus. LPS-treated cells released significantly more O_2^- with each of the three stimuli ($P < 0.001$, paired *t* tests).

§ Addition of LPS (10 ng/ml) at the beginning of the incubation with cytochrome *c*, and preincubation of neutrophils in LPS at concentrations of 1 μg/ml rather than 10 ng/ml gave results almost identical to those shown here (three experiments each).

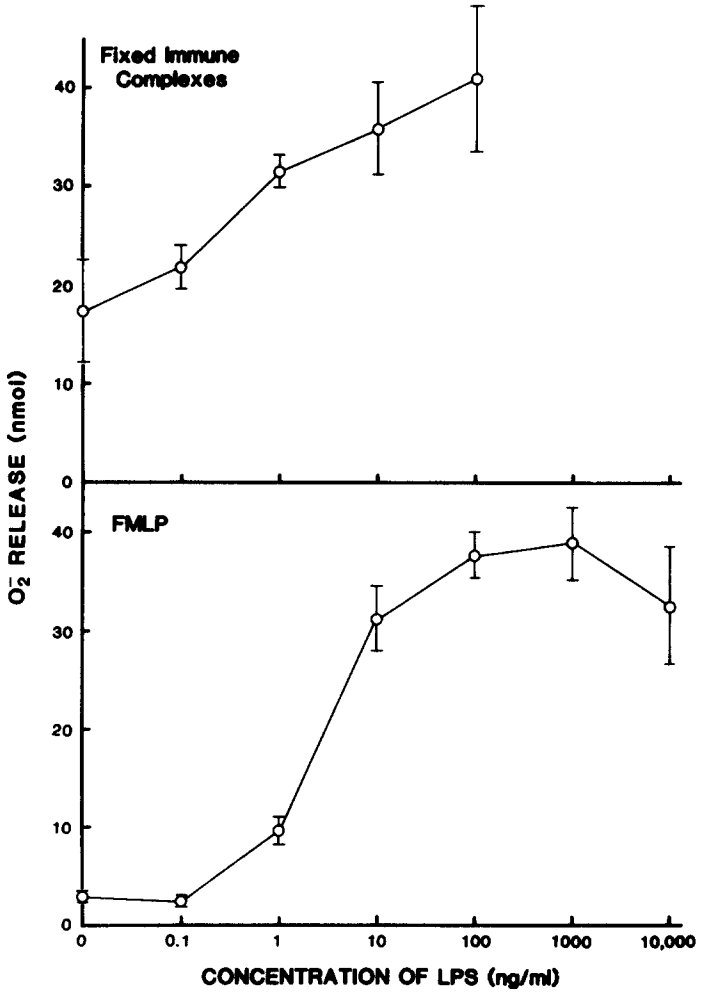

FIGURE 1. Release of O_2^- by neutrophils preincubated with varying concentrations of LPS. The extent of O_2^- release is plotted as a function of the concentration of LPS used in the preincubation with neutrophils for 60 min. The release of O_2^- was stimulated by subsequent exposure of the cells for 60 min to immune complexes fixed to micropore filters or for 10 min to FMLP, 1 μM. The results represent the means ± SEM of three paired experiments with each stimulus.

of LPS, as shown in Fig. 1, in which fixed immune complexes and FMLP were used as stimuli. The priming effect of LPS was variable from donor to donor. An effect at 0.1 ng/ml LPS was observed with cells from only some donors, but priming was consistently and clearly demonstrable at 1 ng/ml and optimal by 10 or 100 ng/ml. Almost identical results for the effect of LPS concentration were obtained in two experiments using 1.5 ng/ml PMA as stimulus (data not shown).

The effect of the duration of preincubation of neutrophils with LPS on the extent of O_2^- release is shown in Fig. 2, using FMLP as stimulus. No significant effect was noted after 15 min of LPS pretreatment, whether FMLP (Fig. 2) or PMA or fixed complexes (not shown) were used subsequently as the stimulus. A

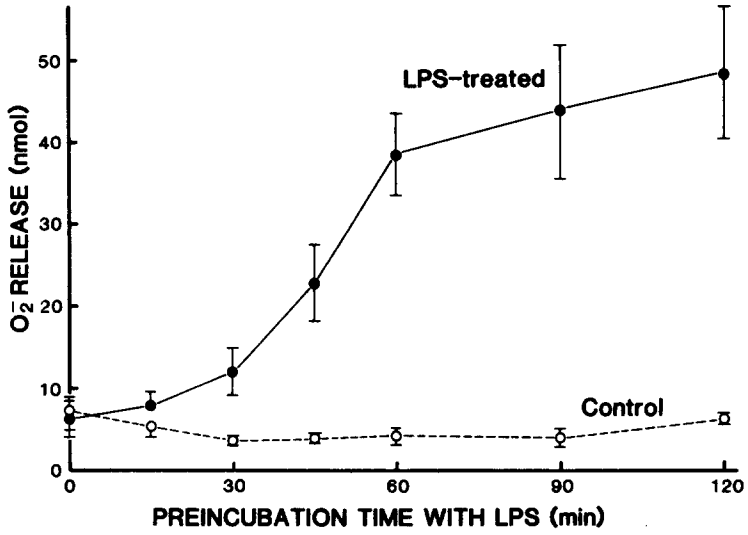

FIGURE 2. Release of O_2^- by neutrophils preincubated for varying lengths of time with LPS, 10 ng/ml, or with buffer alone (control). The release of O_2^- was stimulated by subsequent exposure of the cells to FMLP, 1 μM, for 10 min. Results represent means ± SEM of three paired experiments.

small effect was consistently noted by 30 min, and the effect was prominent by 45 min, whatever the stimulus used. By 60 min of preincubation, priming was ~80% maximal with FMLP (Fig. 2) and 90–100% maximal with PMA or fixed immune complexes (data not shown). The incubation of cells in buffer at 37°C did not alter their O_2^--releasing capacity: freshly prepared cells and cells incubated at 37°C for 60 min released an equivalent amount of O_2^- (29.2 ± 1.8 and 28.6 ± 3.0 nmol, respectively; n = 3, using immune complexes as stimulus).

Although preincubation at 37°C after exposure of cells to LPS was required for priming, the effect could be achieved if the cells were exposed to the LPS at 37°C for as short as 1 min, then centrifuged after 45 s additional time, washed once, and resuspended in a fresh tube in LPS-free buffer for the 60-min preincubation period (mean enhancement 5.2-fold with standard procedure, 4.1-fold with brief exposure; n = 2; 10 ng/ml LPS or FMLP as stimulus). Removal of the LPS and washing would dilute the LPS in the buffer to a concentration below that necessary for priming. Thus, effective association of the LPS with the cells must have taken place during the brief exposure.

Neutrophils pretreated with LPS not only released increased amounts of O_2^- but the release began more quickly and occurred at a faster rate. As shown in Table II, when O_2^- release was monitored continuously, the lag time between addition of the stimulus and appearance of detectable release of O_2^- was decreased markedly after preincubation of the neutrophils in LPS, and the rate of release was faster. The duration of O_2^- release was equivalent for the two cell types using 1 μM FMLP as stimulus (2.8 ± 0.2 min for LPS-treated cells, 2.4 ± 0.2 min for control; mean ± SEM, n = 6, determined as the time until rate increase became negligible). Similar results to those in Table II were obtained in three experiments using fixed immune complexes as stimulus and O_2^- measurement at 5, 7, 10, 15,

TABLE II

*Effect of Priming by LPS on Lag Time and Rate of Release of O_2^-**

Stimulus	Lag time		Rate	
	Buffer	LPS	Buffer	LPS
	s		*nmol/min*	
FMLP, 1 μM	—‡	8 ± 1	3.2 ± 0.8	19.3 ± 1.7
PMA, 1.5 ng/ml	67 ± 12	13 ± 3	13.0 ± 2.0	21.4 ± 3.6
PMA, 0.5 μg/ml	47 ± 7	18 ± 2	17.1 ± 2.1	21.4 ± 3.7
PMA, 1 μg/ml	50 ± 6	15 ± 3	12.6 ± 0.6	21.0 ± 3.7

* Neutrophils (2.5×10^6) were preincubated for 60 min in buffer or buffer plus LPS, 10 ng/ml, then transferred to a cuvette for continuous spectrophotometric monitoring of O_2^- release. The time elapsed between addition of the stimulus and initiation of superoxide dismutase–inhibitable reduction of cytochrome *c* (lag time), and the initial (maximal) rate of cytochrome reduction, were obtained from tracings. Results represent mean ± SEM for three or four paired experiments with each stimulus.

‡ Activity was too low to permit accurate determination of lag time, but values were estimated to be 12–20 s.

20, 30, and 60 min. Detectable release of O_2^- began within the first 5 min for LPS-treated cells (6.8 ± 1.6 nmol; mean ± SEM); no release was detectable from control cells after incubation for 7 min, and release at 10 min was 2.3 ± 0.7 nmol. The maximal rates of release, noted between 0 and 15 min with LPS-treated cells and between 10 and 20 min with control cells, were 1.5 and 1.1 nmol/min, respectively.

Effect of LPS on Release of H_2O_2 and Consumption of Oxygen

The priming effect of LPS was not confined to release of O_2^-. During stimulation with FMLP, neutrophils preincubated with LPS, 10 ng/ml for 60 min, released H_2O_2 at a rate of 7.1 ± 0.5 nmol/min per 5×10^6 cells (mean ± SEM, $n = 10$) compared with a rate of 2.0 ± 0.5 nmol/min for buffer-pretreated cells ($P < 0.001$, paired *t* test). The duration of H_2O_2 release was equivalent in the two cell types (relatively rapid release for about 2.5 min, further slight increase for up to ~5 min). Release of H_2O_2 by unstimulated LPS-pretreated cells was 0.34 ± 0.05 nmol/min and, by unstimulated control cells, 0.36 ± 0.01 nmol/min ($n = 4$).

The rate of FMLP-stimulated oxygen consumption by neutrophils pretreated with LPS was also increased compared with that of control cells (8.1 ± 0.5 and 4.1 ± 0.4 nmol/min per 2.5×10^6 cells, respectively; mean ± SEM, $n = 5$; $P < 0.001$). The duration of oxygen consumption was equivalent for the two cell types (2.8 ± 0.1 min for LPS-treated cells, 2.6 ± 0.1 min for control; time until rate increase became negligible). The extent of enhancement in the rate of oxygen uptake by LPS (1.9-fold) was less than the extent of enhancement of O_2^- release (3.2-fold) in three paired experiments run simultaneously with the same cell preparations.

Investigation of the Molecular Basis of Priming

Effect of temperature, cycloheximide, calcium ion, and serum. The priming event was temperature-dependent. There was no effect if cells were pretreated with

TABLE III
*Effect of Temperature or Cycloheximide on Priming by LPS**

Preincubation temperature	LPS	Cycloheximide	Release of O_2^-‡ (nmol/2.5 × 10^6 neutrophils)
0°C	−	−	6.5 ± 1.7 (3)
0°C	+	−	6.4 ± 1.8 (3)
37°C	+	−	39.2 ± 9.3 (3)
37°C	−	−	7.1 ± 0.9 (4)
37°C	+	−	41.8 ± 2.6 (4)
37°C	+	10 μg/ml	37.0 ± 4.1 (4)

* Neutrophils were preincubated at 0°C or 37°C in buffer or buffer plus LPS, 10 ng/ml, for 60 min before addition of the assay mixture for O_2^-, which included FMLP, 1 μM, as stimulus. Cycloheximide, when present, was preincubated with neutrophils at 37°C for 15 min before addition of the LPS.
‡ Mean ± SEM; the number of experiments is given in parentheses.

LPS at 0°C for 60 min, as shown in Table III, using FMLP as stimulus. Results were similar in two experiments with PMA as stimulus. The presence of cycloheximide at concentrations of 1, 5, 10, 20, or 50 μg per ml 15 min before and during the 60-min preincubation with LPS did not prevent the priming by LPS. Results with 10 μg/ml are shown in Table III. Cycloheximide at this concentration inhibited cellular incorporation of [^{14}C]leucine by >90% (see Materials and Methods).

Priming did not require the presence of calcium ion (Ca^{++}) in the extracellular medium, although absence of Ca^{++} during a 60 min preincubation did reduce the extent of O_2^- release. Neutrophils pretreated for 60 min in buffer without Ca^{++} or Mg^{++}, containing 1 mM EGTA, then assayed for O_2^- generation in the usual buffer (with Ca^{++} and Mg^{++}), released 64.1 ± 0.5 nmol O_2^- for LPS-pretreated cells and 39.7 ± 3.5 nmol for control (60 min pretreatment with 10 ng/ml LPS; n = 2, PMA as stimulus). All of the experiments described were performed without serum present. However, the effect could be demonstrated in the presence of 10% normal human serum (32.5 ± 4.2 nmol for LPS-treated cells compared with 22.9 ± 4.7 nmol for control cells; n = 3, fixed immune complexes as stimulus).

Effect of LPS on binding of FMLP to neutrophils. To explore further the molecular basis for the enhanced oxidative response of LPS-pretreated cells to FMLP, the binding of radiolabeled FMLP was compared in buffer- and LPS-pretreated cells. As summarized in Table IV, the average number of binding sites per cell was slightly, but significantly, decreased in LPS-pretreated cells, and the binding affinity was equivalent. Thus, the enhanced response of LPS-pretreated cells could not be ascribed to the increased binding of the stimulus, at least with FMLP as stimulus.

NADPH-dependent production of O_2^- by neutrophil particulate fractions. The enzyme system believed to be responsible for the initiation of the respiratory burst has been isolated in a membrane-rich subcellular fraction prepared from intact neutrophils stimulated with PMA or opsonized zymosan (14–16). We

TABLE IV

*Effect of Preincubation of Neutrophils with LPS on the Binding of Radiolabeled FMLP**

	Binding of ^{3}H-FMLP‡	
	Neutrophils preincubated with:	
	Buffer	LPS
No. of binding sites/cell	43,200 ± 3,200	29,600 ± 4,400
Affinity (K_d, nM)	14.0 ± 1.2	10.1 ± 3.8

* Cells were preincubated for 60 min with buffer (KRPD) or with KRPD containing LPS, 10 ng/ml, then incubated with F-met-leu-^{3}H phe and processed for cell-associated radioactivity (Materials and Methods).

‡ Mean ± SEM, $n = 4$. The number of binding sites on LPS-pretreated cells was significantly decreased ($P < 0.05$, paired t test). The values for K_d are not significantly different.

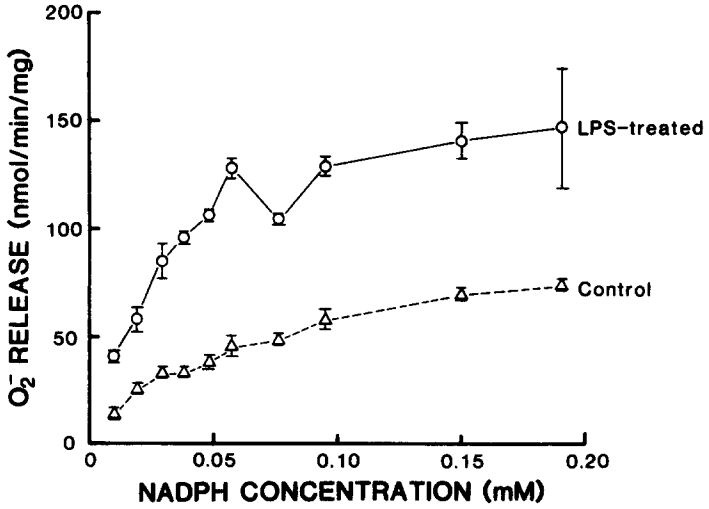

FIGURE 3. Generation of O_2^- by particulate fractions made from neutrophils preincubated with LPS, 10 ng/ml for 60 min, or with buffer alone (control), then stimulated with PMA. The rate of O_2^- release is plotted as a function of the concentration of NADPH in the reaction mixture. Means ± SEM of two experiments are plotted. A third experiment done at different concentrations of NADPH gave almost identical values for the calculated K_m, and the value for V_{max} was three times higher in the fraction from LPS-treated cells. In collated data the V_{max} was significantly increased with fractions from LPS-treated cells ($P < 0.001$, $n = 2$; $P < 0.02$, $n = 3$; paired t tests), using values obtained from analysis of Lineweaver-Burk double reciprocal plots and from a nonlinear regression fit of the data to the hyperbolic Michaelis-Menten equation (17).

explored the possibility that the capacity of LPS pretreatment to enhance the stimulated release of O_2^- might be achieved through an increase in the efficiency or cellular content of this enzyme. In these experiments, enzyme activity was studied as NADPH-dependent production of O_2^-. Particulate fractions were prepared from LPS-treated and control cells stimulated with PMA, or from resting cells. As shown in Fig. 3, the subcellular fraction prepared from LPS-treated neutrophils demonstrated enhanced NADPH-dependent release of O_2^-

over a wide range of NADPH concentrations. Using analysis of Lineweaver-Burk plots, the calculated K_m was the same for fractions from both LPS-treated and buffer-treated neutrophils (45 ± 7 μM and 44 ± 5 μM, respectively; mean ± SEM, n = 3). However, the V_{max} was increased 2.5-fold in the particulate fractions from LPS-treated cells (165 ± 36 nmol/min per mg for LPS-treated fractions and 65 ± 6 nmol/min per mg for controls; n = 3). The particulate fractions from resting (unstimulated) LPS-pretreated and control neutrophils showed minimal O_2^--producing activity (0.6 and 0.4 nmol/min per mg, respectively; means, n = 3). Thus, preincubation with LPS was not sufficient in itself to activate the O_2^--producing enzyme.

Discussion

The data reported here indicate that human blood neutrophils can be primed by exposure to low concentrations of LPS so that they release increased amounts of O_2^- when stimulated. Optimal priming of neutrophils was achieved during ~60 min of incubation with LPS; incubation for >120 min resulted in a slight decline in O_2^- release (data not shown). Mononuclear phagocytes can also be primed with LPS, but optimal priming required incubation for 24 h for macrophages and 48 h for monocytes (4, 5). A pronounced modification like that shown in O_2^- release might have been predicted for mononuclear phagocytes, in view of their known capacity to change size, shape, enzyme content and activity, microbicidal and cytolytic activities, and other characteristics, both in the body and in culture (1). However, the increases in the respiratory burst and in activity of NADPH oxidase in subcellular fractions (Fig. 3) that were induced by exposure of neutrophils to LPS were also pronounced and indicate that the circulating neutrophil is not the metabolically immutable, end-stage cell that it has been generally conceived to be.

In this regard our findings support the conclusions of Granelli-Piperno et al. (2, 3), who found that human neutrophils actively synthesize protein, RNA, and plasminogen activator in vitro. The priming effect achieved by LPS apparently did not require protein synthesis; but it could not be achieved at 0°C and was reduced at room temperature compared with 37°C (data not shown), which is compatible with a requirement for enzymatic activity. Prior exposure to chemotactic factors (C5 fragments, FMLP) also has been shown to enhance neutrophil oxidative metabolic responses and bactericidal activity (reviewed in reference 18), as well as O_2^- production by particulate fractions from stimulated cells (18, 19). It is not clear whether the mechanism of enhancement of the respiratory burst is the same with LPS and chemotactic factors, but the differing requirements for achieving enhancement with the two stimuli suggest that the mechanisms may differ. Optimal priming with FMLP could be achieved in 15 s to 2 min, with subsequent loss of the effect by as early as 10 min; and enhancement was much reduced if the chemotactic factors were removed before addition of the stimulus (18–20; Guthrie and Johnston, unpublished observations). Also, we have found that cytochalasin B, 5 μg/ml, added 5 min before beginning the preincubation with LPS, had no effect on LPS-induced priming, measured as rate of O_2^- release from FMLP-stimulated cells (6.1- and 6.3-fold increases with

and without cytochalasin B, respectively; means, $n = 2$), whereas cytochalasin B significantly enhanced priming induced by chemotactic factors (18, 19).

LPS in our experiments did not appear to act as a stimulus, either by itself or in combination with another, primary stimulus. LPS alone did not cause release of O_2^-, even when added in concentrations up to 100-times higher than those used by us routinely (data not shown). The presence of LPS in the reaction mixture with a stimulus did not increase the extent of O_2^- release induced by that stimulus. Preincubation of cells and LPS at 37°C for at least 30 min was required to achieve a significant enhancement of stimulated O_2^- release, whether the stimulus was FMLP, PMA, or fixed immune complexes. The possibility that LPS might have a direct serum-independent stimulatory effect on neutrophil oxidative metabolism has been investigated previously. Apparently contradictory results have been reported, even from the same laboratory. Higher concentrations of LPS (5–500 μg/ml) than used in our experiments (10 ng/ml) have been shown to directly stimulate hexose monophosphate shunt activity (21–23) and reduction of nitroblue tetrazolium (22–24), but neither of these events is necessarily dependent on the respiratory burst. On the other hand, these concentrations of LPS did not directly stimulate neutrophil generation of O_2^- (22, 23, 25, 26) or chemiluminescence (23), or NADPH oxidizing activity in particulate fractions (22). Results with oxygen consumption have been conflicting (21, 23). Lipid A (5 μg/ml) added to neutrophils settling onto petri dishes has been shown to increase their adherence and concurrent release of O_2^- (25, 26). This effect did not require a preincubation period, but there was an absolute requirement for attachment of the cells to a surface. In experiments probably related to those shown here, preincubation of LPS with rabbit (27) or guinea pig (28) elicited peritoneal neutrophils has been shown to enhance phagocytic bactericidal activity (27) or stimulated release of O_2^- (28).

Studies of the effect of LPS on oxidative metabolism with serum or plasma present in the reaction mixture also have given contradictory results (22, 23, 25, 27, 29). At least some of the contradiction might be due to LPS activation of complement in some systems, with generation of byproducts, especially C5a and $C5a_{desArg}$, that are capable of stimulating oxidative metabolism (30, 31). This stimulatory effect could be modified by the binding and inactivation of the LPS by proteins present in serum (32). Although the priming effect of LPS in our system could be achieved with serum in the incubation mixture, the data reported here were obtained in the absence of serum in order to permit study of the direct interaction between LPS and neutrophils. Priming was achieved in our experiments using concentrations of LPS similar to those detectable by limulus assay in the blood of patients with gram-negative bacteremia (0.5–5 ng/ml) (33).

Inattention to the content of LPS in buffers and reagents used to isolate cells, or in the assays themselves, might also explain some of the discordant results from different laboratories, or even in different assays within the same laboratory. We have found that some lots of commercially available culture medium contain LPS (by limulus assay) in concentrations of 10 ng/ml to 1 μg/ml; that deionized water, commercial animal sera or albumin, and Ficoll solutions usually contain LPS; and that salts used to prepare buffers can become contaminated with LPS once opened. In addition, buffers can leach LPS from micropore filters

(5). In the least, it would seem important to determine the concentration of LPS in all materials used to study the effect of LPS on neutrophils and, in fact, in any materials incubated with neutrophils in assays of their biologic activity. The agents used to elicit cells into the peritoneal cavity of animals, through contaminating LPS or their own stimulatory capacity, also might modify the effects of later exposure of the cells to LPS.

The molecular basis for the priming effect of LPS on neutrophils might involve any of several possible modifications of the cell, none of them mutually exclusive: (*a*) the plasma membrane might be altered so that contact with or binding of the stimuli might be greater; (*b*) the events that couple receptor-stimulus interaction to stimulation of the enzyme responsible for O_2^- production (the oxidase) might be altered, such that more enzyme is activated; or (*c*) the conformation of the enzyme or its placement in the membrane might be changed, such that it expresses higher activity. Our studies suggest that increased binding is not a relevant mechanism, at least with FMLP. Our studies of NADPH-dependent generation of O_2^- by particulate fractions indicate that the K_m of the oxidase for NADPH has not been changed, suggesting that, if modifications of the enzyme or its environment have occurred, they have not influenced interaction of the oxidase with its substrate. However, the V_{max} of the oxidase was increased about 2.5-fold by LPS priming. Since cycloheximide did not inhibit LPS priming, the increased V_{max} is more likely due to more efficient activation of the oxidase than to synthesis of additional enzyme. These results differ from those obtained in studies of the oxidase in normal and activated mouse macrophages: the enzyme from activated macrophages had a decreased apparent K_m for NADPH and slight or no increase in V_{max} (34, 35).

Modifications in stimulus-response coupling (transduction) could be responsible, at least in part, for the enhanced activity of NADPH oxidase induced by LPS. It has been reported that LPS is metabolized by human neutrophils and that in this process the lipid A moiety undergoes deacylation to yield free fatty acids (36). Free unsaturated fatty acids can induce O_2^- release from neutrophils (reviewed in reference 37) and directly activate the neutrophil Ca^{++}- and phospholipid-dependent protein kinase (protein kinase C) (37). This enzyme appears to play a role in signal transduction in a variety of cells (38), and it may be involved in stimulation of the respiratory burst in human neutrophils (37). The time and temperature requirements for LPS priming (30–120 min, optimal at 37°C) are consistent with formation of an LPS metabolite that might stimulate protein kinase C or with stimulation of phosphatidylinositol turnover in the plasma membrane, which could stimulate protein kinase C or, perhaps, prime the cell in some other manner (38). LPS-induced priming could serve as a useful model for the study of signal transduction in neutrophils.

The priming effect of LPS on neutrophils was apparent using three different assay procedures for measuring oxidative metabolism, i.e., O_2^- release, H_2O_2 release, and oxygen consumption. However, the amount of enhancement was lower when oxygen consumption, rather than O_2^- or H_2O_2 release, was measured. Since the assay for oxygen consumption detects only net oxygen uptake, it is possible that one or more of the intracellular reactions that regenerate oxygen

(e.g., dismutation of O_2^- to H_2O_2 and oxygen and breakdown of H_2O_2 by catalase) is increased by exposure of the cell to LPS.

The findings reported here could have clinical relevance. A possible relationship could exist to the neutrophil-mediated inflammatory tissue damage believed to occur in endotoxemic states. One such state might be the adult respiratory distress syndrome associated with infection (39, 40). Experimental models for this syndrome indicate a requirement for neutrophils for the damage to alveolar capillary endothelium and pulmonary interstitium that characterizes the condition (41, 42). The pathogenesis of the syndrome, as currently conceptualized (39), begins with C5a-induced adhesion of neutrophils to lung vascular endothelium. The adherent neutrophils release constituents, including proteases, which can destroy structural proteins, and toxic oxygen metabolites, which can damage endothelial cells (43) and inactivate alveolar proteinase inhibitor (44). The priming effect of LPS shown here could accentuate the destructive capabilities of neutrophils in such a system. Similar considerations might apply to endotoxic shock, in which neutrophils are believed to play an essential role in tissue damage (45).

Summary

We investigated the capacity of bacterial endotoxin (lipopolysaccharide, LPS) to modify the oxidative metabolic response to membrane stimulation of human neutrophils. Neutrophils were pretreated for 60 min with LPS, 10 ng/ml, then stimulated by exposure to fixed immune complexes, the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP), or phorbol myristate acetate. Release of superoxide anion (O_2^-) was up to 7-times greater in cells preincubated with LPS, depending upon the stimulus used. Consumption of oxygen and release of hydrogen peroxide (H_2O_2) were similarly increased, using FMLP as stimulus. The enhancement was accompanied by a reduction in lag time and an increase in the rate of the response, but the duration of the oxidative events was not changed.

The molecular basis for the augmented oxidative response of LPS-pretreated cells was investigated. Preincubation with LPS at 0°C prevented priming, but preincubation in the presence of cycloheximide or chelation of extracellular calcium ion did not. Neutrophils preincubated with LPS had slightly decreased numbers of binding sites and equivalent binding affinity for radiolabeled FMLP. Possible changes in the enzyme responsible for the oxidative burst were analyzed by studying NADPH-dependent generation of O_2^- by particulate fractions from cells preincubated with LPS or buffer, then stimulated before cell disruption. The fraction prepared from LPS-pretreated neutrophils exhibited greater release of O_2^- over a wide range of concentrations of NADPH. The calculated apparent K_m for NADPH was equivalent in the two fractions, but the V_{max} was increased 2.5-fold in the subcellular fraction from LPS-pretreated cells.

These results suggest that LPS could increase neutrophil-mediated host defense or the tissue damage associated with endotoxemia by enhancing the generation of oxygen metabolites by neutrophils. These results also support the concept that the neutrophil is not an end-stage cell in regard to function or metabolic activity.

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