

# Potent CD14-Mediated Signalling of Human Leukocytes by *Escherichia coli* Can Be Mediated by Interaction of Whole Bacteria and Host Cells without Extensive Prior Release of Endotoxin

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**How invading microorganisms are detected by the host has not been well defined. We have compared the abilities of *Escherichia coli* and lipopolysaccharides (LPS) purified from these bacteria to prime isolated neutrophils for phorbol myristate acetate-stimulated arachidonate release, to trigger respiratory burst in 1% blood, and to increase steady-state levels of tumor necrosis factor alpha mRNA in whole blood. In all three assays, bacteria were  $\geq 10$ -fold more potent than equivalent amounts of LPS and could trigger maximal cellular responses at ratios as low as one bacterium per 20 to 200 leukocytes. Both *E. coli* and LPS-triggered responses were enhanced by LPS-binding protein and inhibited by an anti-CD14 monoclonal antibody and the bactericidal/permeability-increasing protein (BPI). However, whereas O polysaccharide did not affect the potency of isolated LPS, intact *E. coli* carrying long-chain LPS (O111:B4) was less potent than rough *E. coli* (J5). Furthermore, material collected by filtration or centrifugation of bacteria incubated under conditions used to trigger arachidonate release or chemiluminescence was 5- or 30-fold less active, respectively, than whole bacterial suspensions. Extracellular BPI (not bound to bacteria) inhibited bacterial signalling, but BPI bound to bacteria was much more potent. Taken together, these findings indicate that *E. coli* cells can strongly signal their presence to human leukocytes not only by shedding LPS into surrounding fluids but also by exposing endotoxin at or near their surface during direct interaction with host cells.**

Mobilization of host defenses against invading bacteria is triggered by signals emanating directly or indirectly from the bacteria. In the case of many gram-negative bacteria, lipopolysaccharides (LPS) provide the most potent signal. Since the bioactive portion of LPS, the lipid A moiety (9, 36, 37), is buried within the outer membrane of intact bacteria, it is generally presumed that shedding of LPS, whether spontaneous during growth or triggered by host factors, is necessary for LPS-mediated signalling of host cells by bacteria (34, 35, 38). Such a model predicts that in the absence of substantial spontaneous or host factor-induced LPS shedding, intact gram-negative bacteria should be relatively ineffective at eliciting host responses. In contrast to this view, we now show potent signalling of host cells by whole *Escherichia coli* without substantial release of LPS-like activity, suggesting that signalling can be mediated by interaction of whole bacteria and host cells. Such cell-cell interaction may promote host cell recognition by delivery of high concentrations of LPS, either by release or increased exposure of envelope endotoxin, in immediate proximity to leukocytes.

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## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacteria used in this study included *E. coli* J5, a rough UDP-galactose-4-epimerase negative mutant (3) (the gift of Loretta Leive, National Institutes of Health, Bethesda, Md.) of the smooth strain O111:B4; *E. coli* K1/r, a K1-encapsulated bacteremic isolate with rough LPS chemotype; *E. coli* O18/K<sup>+</sup> and O18/K<sup>-</sup>, smooth LPS-chemotype strains with and without capsule (kindly provided by Alan S. Cross, Department of Bacterial Diseases, Walter Reed Army Medical Center, Washington, D.C.); *E. coli* O7:K1, a K1-encapsulated strain with smooth LPS-chemotype (ATCC 23503; American Type Culture Collection, Rockville, Md.); and *E. coli* PC2154, a rough nonencapsulated *pldA* K12 derivative (generously provided by Augustus Bekkers, Department of Biochemistry, Center for Biomembranes and Lipid Enzymology, University of Utrecht, Utrecht, The Netherlands). Bacteria were grown in physiological saline supplemented with 0.8% nutrient broth (Difco Laboratories, Detroit, Mich.). Smooth *E. coli* J5 with long-chain LPS was obtained by addition of galactose (0.2 mM) to the growth medium. Overnight cultures were diluted 1:25 into fresh medium and grown to mid-log phase (~3 h) at 37°C. Bacteria were sedimented in a clinical centrifuge at 3,000 × g, washed one or two times, and resuspended in sterile physiological (0.9%) saline to ~10<sup>8</sup> cells per ml. Bacterial concentrations were determined by A<sub>550</sub>, using a Beckman DU-30 spectrophotometer. To radiolabel bacterial proteins during growth, nutrient broth was supplemented with [<sup>35</sup>S]methionine (2.5 μCi/ml at 330 Ci/mmol; DuPont-NEN Research Products, Wilmington, Del.).

**LPS.** Purified LPS from *E. coli* J5 (Rc chemotype; Sigma Chemical Co., St. Louis, Mo.) and *E. coli* O111:B4 (smooth chemotype; Sigma) were resuspended to 1 mg/ml by sonication and vigorous vortexing in sterile pyrogen-free water and stored at 4°C. Immediately prior to use, solutions were sonicated for 2 min either on ice with a probe sterilized by baking at 200°C for  $\geq 4$  h (Ultrasonic Homogenizer 4710; Cole-Palmer Instrument Co., Chicago, Ill.) or in a room temperature water bath (Sonic Dismembrator 550; Fisher Scientific, Springfield, N.J.) at 30% maximal output. Both methods yielded LPS of equivalent bioactivity. Serial dilutions were subsequently made with vigorous vortexing.

**Proteins.** Holo-human bactericidal/permeability-increasing protein (BPI) was purified from polymorphonuclear leukocytes (PMN) as previously described (23, 31). Rabbit LPS-binding protein (LBP) was a generous gift of Peter Tobias and Richard Ulevitch (Department of Immunology, The Scripps Research Institute, La Jolla, Calif.). Human recombinant LBP was provided by Xoma Corp. (Berkeley, Calif.). These two species of LBP had identical effects in our assays. Monoclonal antibody (MAb) directed against CD14 (MY4) and isotype control MAb

(immunoglobulin G2b [IgG2b]) were obtained from Coulter Corp. (Hialeah, Fla.).

**Priming of human PMN.** The release of arachidonic acid (AA) by PMN in response to phorbol myristate acetate (PMA; Sigma) after priming by LPS or bacteria was measured as previously described (2). Heparinized venous blood was obtained from normal volunteers (after informed consent), and PMN were isolated by 3% dextran (pyrogen free; United States Biochemical Corp., Cleveland, Ohio) sedimentation followed by centrifugation in Hybri-max endotoxin-tested Histopaque-1077 (Sigma) or Ficoll-Hypaque (lymphocyte separation medium; Organon Teknika Corp., Durham, N.C.) and hypotonic lysis of erythrocytes. Cells were labeled with [ $^3$ H]AA (60 to 100 Ci/mmol; DuPont-NEN) at  $6 \times 10^4$  or  $6 \times 10^5$  cpm per  $10^8$  PMN in a shaking bath at 37°C for 30 min. After washing twice with 1.5% human serum albumin (HSA; Armour Pharmaceutical Co., Kanakee, Ill.) in Hanks' balanced salts solution without phenol red or divalent cations (HBSS<sup>-</sup>; GIBCO Laboratories, Grand Island, N.Y.) to prevent aggregation of PMN, cells were resuspended in HBSS<sup>-</sup> to  $2.5 \times 10^7$  cells per ml and incubated at 37°C for an additional 10 min of chase. HSA was then added to 1.5%. LBP, MY4, or IgG2b, where applicable, was added to PMN suspensions immediately prior to the addition of LPS, bacteria, or supernatants or filtrates thereof. A single *E. coli* cell contains  $\sim 2 \times 10^6$  molecules of LPS (11, 29, 33, 40), which corresponds to  $\sim 10$  ng of J5 LPS per  $10^6$  cells. The cell suspension was incubated in most experiments for 45 to 60 min (without LBP), 15 min (with 0.75  $\mu$ g of rabbit LBP per ml or with 1  $\mu$ g of human LBP per ml), or for the times indicated. After this incubation, Ca<sup>2+</sup> and Mg<sup>2+</sup> salts were added to final concentrations of 1.26 and 0.9 mM, respectively, PMA was added to a final concentration of 33 ng/ml, and the cell suspension was incubated for an additional 15 min at 37°C in a shaking bath. Release of [ $^3$ H]AA and metabolites was measured by sedimenting the cells at 14,000  $\times$  g for 5 min at 4°C and counting a portion of the recovered supernatant in a Beckman LS5000TD liquid scintillation counter, using Ecocint A (National Diagnostics, Atlanta, Ga.). The percentage of radiolabeled fatty acids released from esterified lipids in response to LPS or bacteria and subsequent stimulation with PMA was determined as [cpm (supernatant) - cpm (supernatant of non-PMA-stimulated cells)/cpm (total) - cpm (supernatant of non-PMA-stimulated cells)]  $\times$  100. Except for the data in Fig. 1, the percent release induced by PMA alone (no priming) was also subtracted.

**Chemiluminescence in diluted blood.** Chemiluminescence induced by added *E. coli* or purified LPS in 1% human blood (final concentration) was measured by using lucigenin (bis-*N*-methylacridinium nitrate; Sigma) as a probe as previously described (28). Blood from consenting human volunteers was collected (without anticoagulant) and immediately diluted to 1.1 or 1.5% in Hanks' balanced salts solution without phenol red containing 1.26 mM Ca<sup>2+</sup> and 0.8 mM Mg<sup>2+</sup> (HBSS<sup>+</sup>; Whittaker Bioproducts, Walkersville, Md.) buffered with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4). Final sample volume was 1 ml; lucigenin was added to a final concentration of 30  $\mu$ g/ml (59  $\mu$ M). MY4 or IgG2b, where applicable, was added immediately before stimulant in a volume of 2 to 10  $\mu$ l. Samples containing 100 or 300  $\mu$ l of stimulant (LPS, bacteria, or supernatants or filtrates thereof) were added last to 900 or 700  $\mu$ l of 1.1% blood-lucigenin or 1.5% blood-lucigenin, respectively, and chemiluminescence was monitored in the dark for up to 10 h (at 30-min intervals), using a Beckman LS5000TD liquid scintillation counter operated in single-photon mode and set to count each sample for 0.5 min. Chemiluminescence units (CLU) were derived as follows:  $CLU = 20(1/t)^2 + m/(2 \times 10^6)$ , where *t* is time (in hours) at which peak chemiluminescence is reached and *m* is maximal chemiluminescence. This datum transformation was developed to convert time curves of chemiluminescence activity of a range of J5 LPS doses (in several different experiments) into roughly logarithmic LPS dose curves and to facilitate quantitative comparison of the activities of different samples. In all experiments presented, differences in chemiluminescence-inducing activity correspond to differences in both the magnitude and kinetics of induced chemiluminescence.

**TNF- $\alpha$  gene expression in whole blood.** Levels of tumor necrosis factor alpha (TNF- $\alpha$ ) mRNA were measured as described by Dedrick and Conlon (1), with several modifications. LPS (0.1 to 2.0 ng/ml) or bacteria ( $10^4$  to  $2 \times 10^5$  cells per ml) were either incubated in citrated human venous blood at 37°C for 1 or 5 h or preincubated in citrated fresh human plasma at 37°C for 0, 1, or 2 h before incubation with citrated fresh human venous blood at 37°C for an additional 1 h. Incubations were terminated by lysis-homogenization of 75  $\mu$ l of each sample with 1 ml of Ultraspec RNA reagent (Biotex Laboratories, Inc., Houston, Tex.). RNA was isolated by chloroform extraction and isopropanol precipitation and was washed twice with ethanol according to the manufacturer's instructions. RNA was converted to cDNA by using a first-strand cDNA synthesis kit (Pharmacia) employing a *NotI*-d(T)<sub>18</sub> primer. TNF- $\alpha$  cDNA was amplified by PCR using the following primers: 5'-CCT CCT CAC AGG GCA ATG ATC CCA-3' and 5'-AGT GAC AAG CCT GTA GCC CAT GTT G-3'. Control glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA was amplified by using 5'-CGG GGC TCT CCA GAA CAT CAT CC-3' and 5'-CCA TGA GGT CCA CCA CCC TGT TG-3' (generously supplied by Russell L. Dedrick, Xoma Corp.). The amplified product was extracted with CHCl<sub>3</sub> and separated by 1.5% agarose gel electrophoresis. Bands were visualized by staining with ethidium bromide and exposure to UV light. PCR product band densities for both TNF- $\alpha$  and G3PDH diminished in a dose-dependent fashion with decreasing amounts of cDNA added as the template.

**cPLA<sub>2</sub> assay.** The alteration of the electrophoretic mobility of the 85-kDa cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) of PMN in response to LPS or bacteria was determined as previously described (2).

**Preparation of filtrates and supernatants for LPS shedding assays.** LPS or bacteria were incubated at a concentration of 10 ng/ml or  $10^6$  cells per ml, respectively, under priming conditions (HBSS<sup>-</sup> containing 1.5% HSA, 37°C for 15 to 30 min) or chemiluminescence conditions (1% plasma in HBSS<sup>+</sup>, 25°C for 1 h; 1% plasma does not inactivate LPS or bacteria in 1 h). Surfactant-free cellulose acetate membranes (0.45  $\mu$ M; Nalge Co., Rochester, N.Y.) were pre-washed with HBSS<sup>-</sup> containing 1.5% HSA before filtration of bacteria or LPS suspensions. Under these conditions,  $\geq 85\%$  of radiolabeled LPS (at 10 ng/ml) from *E. coli* LCD25 (generously provided by Robert S. Munford, Departments of Microbiology and Internal Medicine, University of Texas Southwestern Medical Center, Dallas) is recovered in the filtrate. Supernatants were prepared by centrifugation for 10 min at 14,000  $\times$  g.

**Assay for BPI effects on chemiluminescence-inducing activity of *E. coli* and purified LPS.** LPS (10 ng/ml) or *E. coli* J5 ( $10^6$  cells per ml) was preincubated with BPI for 1 h at 37°C with shaking in either HBSS<sup>+</sup> or HBSS<sup>-</sup> buffered with 10 mM HEPES (pH 7.4). Samples were diluted 10-fold into 1% blood for measurement of chemiluminescence. To determine the contribution of bound BPI to inhibition of *E. coli*-induced chemiluminescence, <sup>35</sup>S-labeled *E. coli* cells ( $5 \times 10^7$ /ml) were preincubated with BPI (300 nM) in either HBSS<sup>+</sup> or HBSS<sup>-</sup> buffered with 10 mM HEPES (pH 7.4) (the higher bacterial concentration was used to improve recovery of BPI-treated bacteria during subsequent wash steps). Unbound BPI was removed by centrifugation for 5 min at 14,000  $\times$  g followed by one wash in either HBSS<sup>+</sup> or HBSS<sup>-</sup> buffered with 10 mM HEPES (pH 7.4). Chemiluminescence-inducing activity of bacteria, before and after removal of unbound BPI, was measured as described above. Bacterial recovery after washing was monitored by liquid scintillation counting using Aquasol-2 (Packard Instrument Co., Meriden, Conn.).

## RESULTS

**Added live *E. coli* cells are more potent inducers of host cell responses than equivalent amounts of purified LPS.** To determine the relative potency of bacteria and LPS in signalling PMN, [ $^3$ H]AA-labeled isolated human PMN were treated with increasing amounts of either *E. coli* J5 or LPS purified from *E. coli* J5. At the doses tested, neither LPS (not shown) nor *E. coli* (Fig. 1A) directly activated the PMN but rather primed these cells for greater release of [ $^3$ H]AA during subsequent stimulation with PMA (Fig. 1A). On the basis of the amount of LPS needed to trigger priming of PMN, *E. coli* suspensions were  $\sim 10$ -fold more potent than isolated LPS. Whole *E. coli* were also more potent than purified LPS in induction of the respiratory burst (measured by chemiluminescence) in 1% blood, causing earlier and higher peak production of reactive oxygen species (Fig. 1B to D). In addition, whole *E. coli* caused more prolonged signalling than purified LPS in whole blood, as measured by TNF- $\alpha$  mRNA elevation (Fig. 2A). At LPS concentrations of  $< 10$  ng/ml, LPS induces elevation of steady-state TNF- $\alpha$  mRNA levels in whole blood ex vivo that is short-lasting and peaks 1 h after exposure of whole blood to LPS (1) (Fig. 2A, lanes 7 and 8). The transient nature of LPS induction of TNF- $\alpha$  mRNA elevation parallels plasma-mediated LPS neutralization (1, 61, 62) (Fig. 2B, lanes 5 to 7). In contrast, both *E. coli* that is rapidly killed (J5) and to a greater extent a strain that survives and multiplies in whole blood (O7:K1) caused more prolonged elevation of TNF- $\alpha$  mRNA (Fig. 2A, lanes 3 to 6) and were much less susceptible to inactivation by plasma (Fig. 2B, lanes 2 to 4). Levels of G3PDH mRNA were unaffected by LPS or *E. coli* (Fig. 2A and B, bottom panels). Thus, under a wide range of conditions and functional responses, live *E. coli* appeared to be much more potent stimuli of host responses than the LPS purified from these organisms.

**One bacterium can trigger the response of more than 20 PMN.** A maximal priming response occurred with  $10^5$  or  $10^6$  *E. coli* J5 cells and  $2 \times 10^7$  PMN per ml, i.e., one bacterium per 20 to 200 PMN. Only 10 to 20% of the cellular arachidonic pool was released following stimulation with PMA under these conditions, raising the possibility that only a fraction of the PMN population was stimulated. However, during pri-

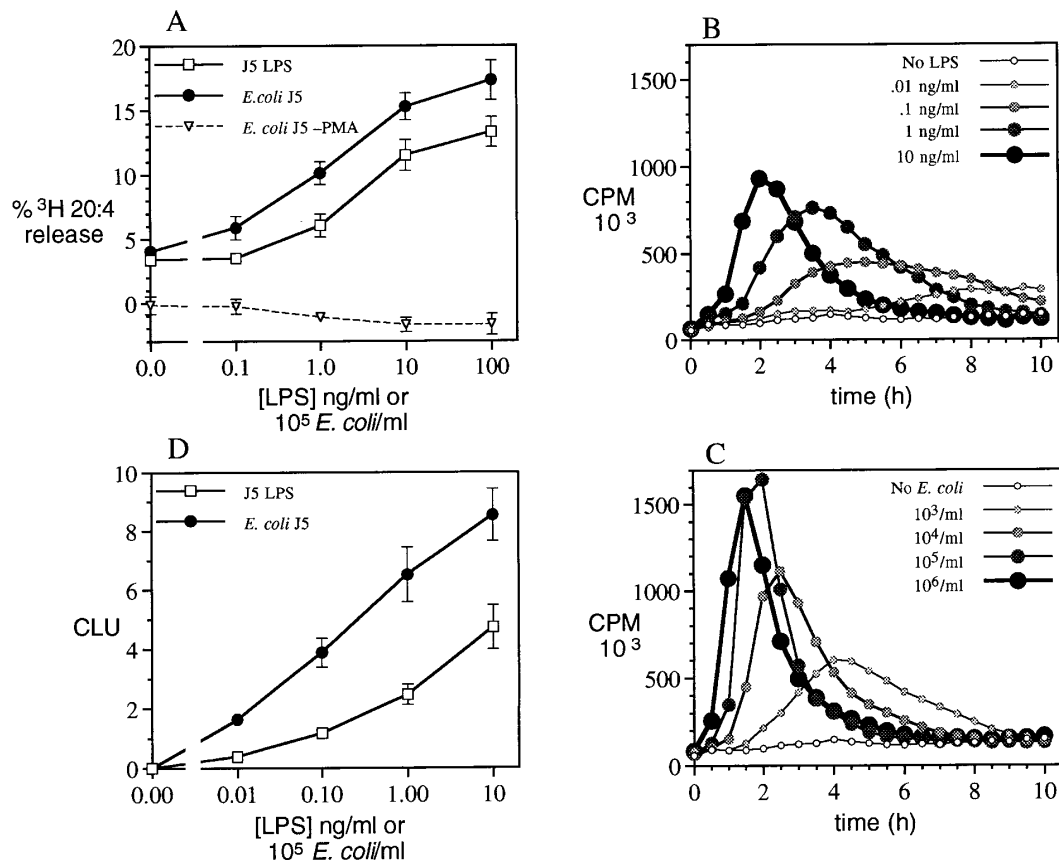


FIG. 1. Comparison of the signalling activities of whole *E. coli* J5 and equivalent amounts of LPS purified from *E. coli* J5 (see Materials and Methods). (A) Priming of labeled human PMN ( $2 \times 10^7$ /ml in HBSS<sup>-</sup> containing 1.5% HSA) for enhanced release of [<sup>3</sup>H]AA (20:4) in response to PMA by preincubation with *E. coli* or LPS for 60 min was determined as described in Materials and Methods. Results represent the mean  $\pm$  standard error of the mean of  $\geq 4$  independent experiments. Dose- and time-dependent chemiluminescence of 1% blood in response to J5 LPS (B) and *E. coli* J5 (C) was measured by lucigenin-enhanced chemiluminescence as described in Materials and Methods. (D) Representation of data from panels B and C as CLU (see Materials and Methods). Results represent the mean ( $\pm$  standard error of the mean in panel D) of six independent experiments.

mary with bacteria as well as with LPS nearly all of the PMN cPLA<sub>2</sub> was converted to a more slowly migrating form, as visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3). This result demonstrates that essentially all PMN are primed by *E. coli* J5 at ratios of one bacterium per 20 to 200 PMN.

**Role of LBP and CD14 in bacterial signalling.** Signalling of PMN by purified LPS is mediated by binding of LPS to membrane CD14 and is markedly enhanced by complexing of LPS with the plasma LPS-binding protein LBP (2, 44, 45, 47, 49, 51,

59, 60) (Fig. 4). To determine whether signalling by bacteria is also largely mediated by this pathway as suggested by Landmann et al. (21), we tested the effects of LBP and the neutralizing anti-CD14 Mab MY4 on bacterium-triggered priming of PMN. Signalling of PMN by added live *E. coli* J5 is potentiated by LBP and is CD14 dependent (Fig. 4 and 5). At a relatively low dose of purified LPS (0.1 ng/ml), LBP increased the magnitude of priming, whereas at a higher dose of LPS (100 ng/ml) and at either  $10^4$  or  $10^6$  *E. coli* cells per ml, LBP accelerated the priming response (Fig. 4). The kinetics of priming of PMN

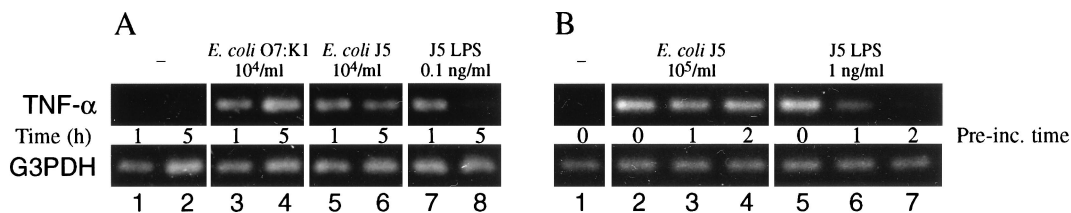


FIG. 2. Whole bacteria cause more sustained elevation of TNF- $\alpha$  mRNA levels in blood cells than does purified LPS, paralleling faster inactivation of LPS by plasma. (A) Whole blood was incubated for 1 h (lanes 1, 3, 5, and 7) or 5 h (lanes 2, 4, 6, and 8) with no stimulus (lanes 1 and 2),  $10^4$  *E. coli* O7:K1 cells per ml (lanes 3 and 4),  $10^4$  *E. coli* J5 cells per ml (lanes 5 and 6), or 0.1 ng of J5 LPS per ml (lanes 7 and 8). Levels of TNF- $\alpha$  and G3PDH mRNA were determined as described in Materials and Methods. (B) Levels of TNF- $\alpha$  and G3PDH mRNA were measured in reconstituted whole blood incubated for 1 h with LPS or *E. coli* that had been preincubated in plasma at 37°C for 0, 1, or 2 h before addition to blood cells. Lane 1, no stimulus; lanes 2 to 4,  $10^5$  *E. coli* J5 cells per ml; lanes 5 to 7, 1 ng of J5 LPS per ml. Results are representative of at least two separate experiments. Within 15 min of incubation in plasma or whole blood,  $>99\%$  of added *E. coli* J5 cells were killed. In contrast, levels of viability of *E. coli* O7:K1 were 130% and  $\geq 1,000\%$  of the inoculum at 1 and 5 h, respectively.



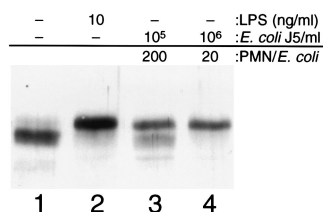


FIG. 3. cPLA<sub>2</sub> from PMN is quantitatively converted to a more slowly migrating form during priming with one *E. coli* cell per 20 PMN. cPLA<sub>2</sub> was immunoprecipitated from PMN cytosol and detected by immunoblotting as described previously (2). Lane 1, cPLA<sub>2</sub> from unprimed PMN; lanes 2 to 4, cPLA<sub>2</sub> from PMN primed for 60 min with the indicated concentrations of LPS or bacteria. Results shown represent one of two closely similar experiments.

by *E. coli* and by purified LPS were essentially the same. Pre-treatment of PMN with MY4, but not with an isotype-matched control antibody, virtually abolished priming of PMN by low doses of *E. coli* J5 in the presence or absence of LBP (Fig. 5). MY4 also inhibited priming of PMN by higher doses of bacteria (10<sup>6</sup> *E. coli* cells per ml) nearly completely in the presence of LBP but incompletely in its absence. In addition, MY4 produced nearly complete inhibition of LPS and bacterium-induced chemiluminescence (data not shown).

**Shedding of bacterial constituents accounts for only a small fraction of the signalling activity of *E. coli*.** Signalling of many PMN by a single bacterium could reflect release of bacterial components (e.g., LPS). However, cell-free supernatants and filtrates collected after incubation of *E. coli* J5 for up to 30 min in the medium used for priming assays exhibited only ~20% of the activity of the whole bacterial suspension and of the recovered bacterial pellet (Fig. 6A). Inclusion of PMN in the incubation mixture did not increase activity in the filtrate (data not shown). Bacterial filtrates collected after incubation of *E. coli* for up to 1 h in 1% plasma (Fig. 6B) or 1% blood (data not shown) also contained ≤10% of the activity of the bacterial suspension. In contrast, more than 80% of the activity of added purified LPS was recovered in the extracellular supernatants and filtrates under these conditions. Thus, signalling of PMN by *E. coli* does not appear to be mediated by bacterial or host cell products released either constitutively or during interaction of bacteria and host cells.

**Effect of LPS O antigen on signalling by bacteria.** The inability of shed material to account quantitatively for the signalling activity of bacterial suspensions suggested that signalling by *E. coli* may depend on direct contact between bacteria

and leukocytes. To test this possibility, we examined the effect of the presence of LPS O antigen on bacterial signalling, since additional polysaccharides impede interaction between bacteria and leukocytes (17, 50, 54) but not interactions with cell-free LPS (20). Conversion of *E. coli* J5 to a smooth phenotype (identical to that of the parent strain *E. coli* O111:B4) by growth in galactose-containing media caused a ~100-fold reduction in bacterial signalling activity in the PMN priming assay (Fig. 7A) and a ~10-fold reduction in activity in the chemiluminescence assay (data not shown). Similar differences in activity were also seen with other rough (PC2154 and K1/r) and smooth (O18K<sup>+</sup> and O18K<sup>-</sup>) strains of *E. coli* (not shown). In contrast, no difference was detected in the potencies of approximately comparable molar concentrations of purified J5 LPS and smooth O111:B4 LPS in these assays (Fig. 7B).

**Comparison of BPI-mediated inhibition of signalling by *E. coli* and purified LPS.** The experiments described above suggest that direct bacterium-host cell interaction is required for potent LPS-dependent bacterial signalling in both the priming and chemiluminescence assays. To further address this possibility, we tested the ability of BPI, a bactericidal and LPS-neutralizing protein of PMN (22, 26, 28, 30, 52), to inhibit signalling by *E. coli* under conditions allowing more or less binding of BPI to the bacteria. Although Ca<sup>2+</sup> and Mg<sup>2+</sup> do not, at physiological extracellular concentrations, affect BPI-mediated inhibition of purified LPS-triggered signalling (Fig. 8A), they are important determinants of bacterial binding and antibacterial action of BPI (reference 5 and unpublished data). The inhibitory potency of BPI toward bacterium-mediated signalling was 10- to 100-fold greater when BPI was preincubated with *E. coli* in the absence of divalent cations than when it was preincubated in the presence of divalent cations (Fig. 8A). This greater inhibitory effect of BPI in the absence of divalent cations paralleled a ~20-fold greater bacterial binding of BPI (not shown). Moreover, when bacteria were pretreated with BPI in the absence of divalent cations, removal of unbound BPI by washing *E. coli* only slightly reduced the inhibitory effect of BPI; removal of unbound BPI after preincubation in the presence of divalent cations virtually eliminated inhibition by BPI of bacterial signalling (Fig. 8B). Thus, in these experiments both bound and unbound BPI could inhibit bacterial signalling, but the potency of BPI was greatly enhanced by conditions that promoted binding of BPI to the bacterial surface.

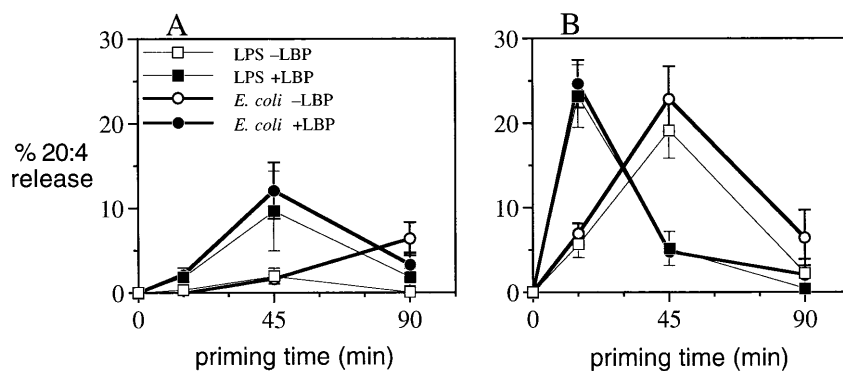


FIG. 4. LBP potentiates the PMN priming activity of both *E. coli* J5 and LPS. Labeled PMN ( $2 \times 10^7$ /ml) were preincubated with low doses of LPS (0.1 ng/ml) or *E. coli* J5 (10<sup>4</sup> cells per ml) (A) or high doses of LPS (100 ng/ml) or *E. coli* J5 (10<sup>6</sup> cells per ml) (B) for the times indicated in the presence (solid symbols) or absence (open symbols) of LBP (0.75 to 1.0 μg/ml) before addition of PMA and measurement of [<sup>3</sup>H]AA release as described in Materials and Methods. Data represent the mean ± standard error of the mean of three independent experiments.

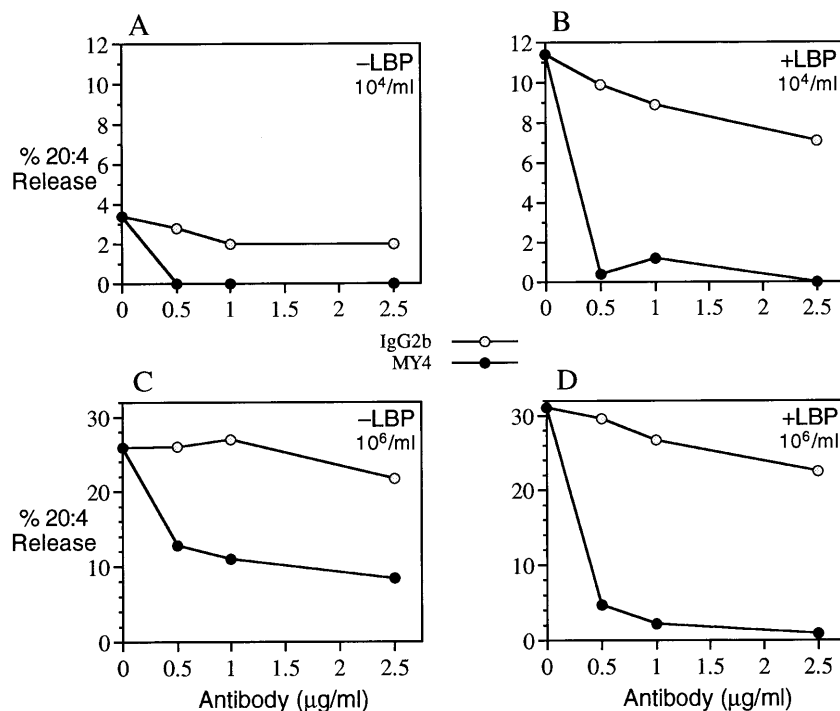


FIG. 5. Inhibitory effect of the anti-CD14 monoclonal antibody MY4 on *E. coli*-induced priming of PMN in the presence (B and D) or absence (A and C) of LBP (0.75 µg/ml). MY4 or an isotype IgG2b control antibody, in the doses indicated, was added to PMN suspensions just prior to the addition of *E. coli*. *E. coli* was added to a final concentration of  $10^4$  (A and B) or  $10^6$  (C and D) cells per ml. Preincubation times (of *E. coli* with PMN) were selected to yield maximal response in each case: 90 min (A), 45 min (B and C), and 15 min (D). Results shown are from one experiment representative of at least two similar experiments.

## DISCUSSION

The nature of the exquisite sensitivity of many animal species (including humans) to minute quantities of bacterial endotoxin has been under intense scrutiny for decades. Recently, great strides have been made in the elucidation of pathways by which the host can detect systemically introduced envelope LPS of gram-negative bacteria. The use of purified LPS has led to the identification and isolation of two LPS-binding proteins that have been shown to play essential roles in host cell responses to LPS, i.e., the circulating acute-phase LBP (10, 39, 47, 48) and CD14, a leukocyte-bound glycoprotein that also exists in soluble extracellular form (14–16, 44, 49, 60). LBP

promotes the delivery of LPS to cells that carry plasma membrane-linked CD14, an LPS receptor molecule that is a critical component of the apparatus that transmits the LPS signal to intracellular biochemical response systems (45, 46, 63). Leukocyte membrane-anchored CD18/CD11 integrins can also bind LPS (13, 55–58) and mediate LPS signalling (18) but require higher LPS levels than the CD14 pathway and are inhibited rather than enhanced by plasma proteins such as LBP. In addition to these positive regulators of host responses to LPS, other LPS-binding proteins provide the host with means of dampening the LPS signal (for reviews, see references 4, 5, 7, and 8). The finding that administered purified

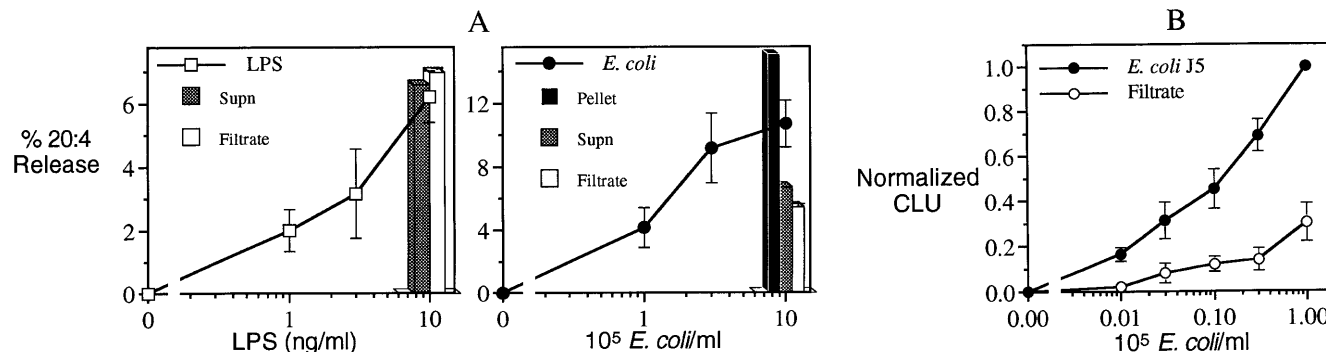


FIG. 6. Shedding of bacterial constituents accounts for only a small fraction of the signalling activity of *E. coli*. J5 LPS (10 ng/ml) or *E. coli* J5 ( $10^6$  cells per ml) was incubated at 37°C in HBSS<sup>-</sup> containing 1.5% HSA for 30 min to mimic priming conditions (A) or at 25°C in HBSS<sup>+</sup> containing 1% plasma for 1 h to simulate chemiluminescence conditions (B). After incubation, extracellular supernatants (Supn) and filtrates were collected as described in Materials and Methods and tested in comparison with suspensions of whole bacteria and purified LPS for priming activity (A) and chemiluminescence activity (B). Note that the units on the vertical axis of the right panel are twice those on the left panel. Results represent the mean  $\pm$  standard error of the mean of at least two independent experiments. To facilitate quantitative comparison of different experiments, the CLU activity of  $10^5$  *E. coli* cells per ml was normalized to a value of 1.0 in each experiment.

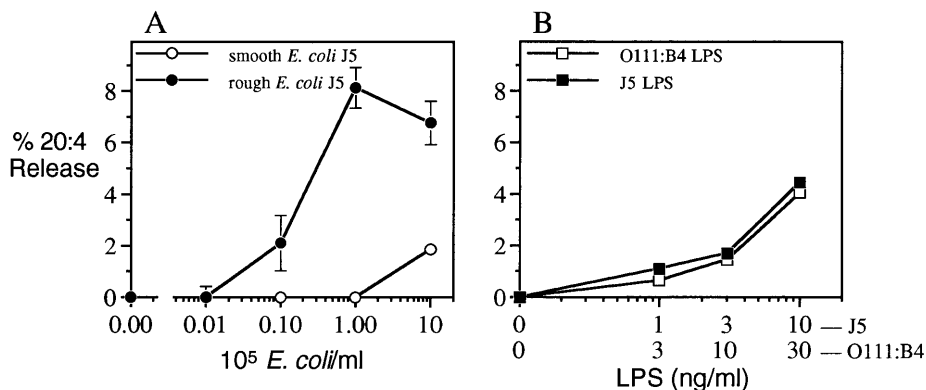


FIG. 7. Effect of LPS O antigen on signalling by bacteria. (A) Comparison of AA release after 45 min of priming of PMN (see Materials and Methods) by *E. coli* J5 grown without (rough chemotype) and with (smooth chemotype) 0.2 mM galactose. Results represent the mean  $\pm$  standard error of the mean of at least two experiments. (B) Dose-dependent priming by LPS purified from rough *E. coli* J5 and from its smooth parent *E. coli* O111:B4. Results represent the mean of two experiments. The scale on the abscissa is arranged so that the molar amounts of J5 and O111:B4 LPS added are approximately equal.

LPS mimics the endotoxic effects of invading gram-negative bacteria (9, 34, 36, 37) has led to the assumption that the host recognizes gram-negative bacteria by detecting their LPS.

It appears likely that in our experiments, responses to bacteria are indeed mediated principally by LPS because bacterial signalling is augmented by LBP (Fig. 4) and is blocked by a MAb against CD14 (Fig. 5) (21) and by BPI (Fig. 8A), a highly specific inhibitor of LPS bioactivity (6, 12, 26, 52). The incomplete blocking by an anti-CD14 MAb of signalling by large numbers of bacteria in the absence of LBP (Fig. 5C) might be explained by the presence of the complement receptor CR3 (CD18/CD11b) on PMN (13).

However, the results of this study challenge the prevailing notion that host defense cells detect invading gram-negative bacteria primarily by recognizing LPS molecules shed into the surrounding body fluids. Stimulation of host cell responses by purified LPS and that by whole bacteria are distinctly different.

Intact *E. coli* J5, a strain bearing short-chain LPS in its envelope, triggers more vigorous (AA release and respiratory burst activity [Fig. 1]) or more extended (TNF- $\alpha$  mRNA levels [Fig. 2]) responses when added to human leukocytes incubated in artificial medium, 1% blood, or undiluted blood, respectively, than do equivalent amounts of isolated LPS extracted

from the same *E. coli* strain. The higher activity of whole bacteria suggests that very effective signalling is possible either by direct contact between bacteria and host cells or if the bacteria shed a material with potency much greater than that of purified LPS.

Considerable shedding of endotoxin-like activity by *E. coli* has been reported during growth for 16 h (27). However, such shedding cannot account for the potent signalling that we have observed within 15 to 45 min (AA release) or  $\sim$ 2 h (respiratory burst activity) after addition of bacteria. Tesh et al. have reported complement-dependent release of LPS from *E. coli* (which appears to be less extensive with rough than smooth organisms) (41, 42), but the activity of this released LPS was low, presumably because of inactivation by serum proteins (43). Moreover, complement is not necessary for the bacterial signalling that we have observed (serum is absent in the PMN priming assay). Most important, the signalling activity of bacterial constituents released extracellularly during preincubation of the bacteria in artificial medium or 1% plasma accounts for at most 20% of the activity of the whole bacterial suspension (Fig. 6). Incubation of bacteria in the presence of 1% blood or PMN at various ratios does not increase the signalling activity of filtrates or cell-free supernatants of the suspensions

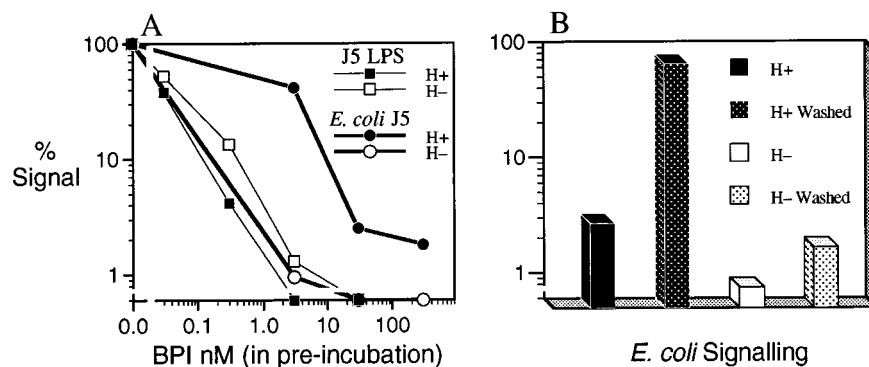


FIG. 8. Inhibition of LPS and *E. coli*-induced chemiluminescence by BPI. (A) Effect of increasing doses of BPI preincubated with J5 LPS (10 ng/ml) or *E. coli* J5 ( $10^6$  cells per ml) in HBSS in the presence ( $H^+$ ) or absence ( $H^-$ ) of  $Ca^{2+}$  and  $Mg^{2+}$ . Chemiluminescence-inducing activity of purified LPS or bacteria preincubated with BPI was calculated relative to standard curves of LPS or bacteria. Results represent the mean of two separate experiments, each done in duplicate. (B) Effect of bound and unbound BPI on chemiluminescence-inducing activity of *E. coli* J5. *E. coli* ( $5 \times 10^7$  cells per ml) was preincubated with 300 nM BPI in HBSS $^+$  ( $H^+$ ) or HBSS $^-$  ( $H^-$ ). Chemiluminescence-inducing activity of BPI-treated bacteria was measured before and after washing of bacteria to remove unbound BPI (as described in Materials and Methods) and calculated relative to the standard curve of untreated *E. coli*. Data shown are from one experiment done in triplicate representative of two similar experiments.

(not shown). Kelly et al. (20), using rough mutants of *Salmonella* strains, also observed limited filtrable LPS activity after 4 h in HEPES-buffered HBSS. That host cell responses may be triggered by direct interaction with whole bacteria is further suggested by the recent report (19) that the LPS receptor CD14 is necessary and sufficient for binding of *E. coli* by human monocytes.

To explain potent signalling by bacteria without substantial release of LPS into the medium, we propose that close contact between bacteria and host cells generates strong LPS signals, possibly further amplified by other envelope constituents. Such signals may be delivered by a small shed fraction of the bacterial LPS content that reaches high local concentrations in immediate proximity to leukocytes or by LPS still within the envelope, but possibly more exposed following host-induced bacterial surface alterations. According to this scheme, direct contact between host cells and whole bacteria permits more efficient delivery of signals than shedding of bacterial constituents (LPS) in more dilute form into the surrounding fluids.

Such a scheme is also consistent with the finding that BPI inhibits signalling more effectively when bound to the bacteria than when not bound (Fig. 8). BPI in the fluid phase may not have sufficient access to locally released LPS (as opposed to its ability to interact with added purified LPS) to compete effectively with LPS binding to host cell CD14. In addition, when conditions for bacterial binding of BPI are unfavorable, continued direct host cell-bacterium contact and signalling may also account for reduced BPI effectiveness under those conditions. It should be stressed that while physiologic concentrations of extracellular divalent cations do reduce BPI binding to bacteria, these effects can be overcome in body fluids (e.g., plasma and inflammatory fluids) in which synergy with other host proteins (such as complement) promotes BPI interactions with gram-negative bacteria (references 22, 25, 52, and 53 and unpublished observations).

*E. coli* J5 is not killed either in HBSS or in 1% blood (not shown), and therefore phagocytosis need not be considered as a likely trigger for stimulated AA and O<sub>2</sub> metabolism under these conditions. However, in whole blood, substantial complement-mediated killing and damage as well as phagocytosis of this relatively fragile organism may be expected. In contrast, the highly serum- and phagocytosis-resistant encapsulated smooth *E. coli* O7:K1 actually multiplies in whole blood and, in contrast to the rough *E. coli* J5, is therefore unlikely to undergo prominent envelope changes (LPS release). Nevertheless, not only the more degradable *E. coli* J5 but also *E. coli* O7:K1 induces more sustained levels of TNF- $\alpha$  mRNA in host cells than does purified LPS (Fig. 2), consistent with signalling by intact bacteria. It has been suggested that the transient induction of the TNF- $\alpha$  message in host cells by purified LPS is due to LPS-inactivating mechanisms in whole blood or plasma (1). Less susceptibility of whole bacteria to such inactivation may explain the more sustained levels of TNF- $\alpha$  mRNA, and/or continued transmission of signals may occur during contact with host cells. Smooth *E. coli* strains stimulate AA metabolism in HBSS 10- to 100-fold less potently than do *E. coli* strains devoid of O-antigen-containing LPS, while isolated long- and short-chain LPS do not differ in signalling activity (Fig. 7) (20). Under these less physiologic conditions, such reduced stimulation by smooth strains may reflect less intimate contact with leukocytes, or during contact, these more host-resistant strains may undergo less pronounced envelope changes required for signalling.

Our finding that a single bacterium is able to stimulate many host cells (Fig. 3) remains unexplained. Attachment of bacteria to host phagocytes is facilitated by circulating host factors and

is a necessary step for subsequent intracellular sequestration. The strength of bacterial attachment to host cells, mediated by CD14 and other molecules (19, 32), and whether or not fleeting contact is sufficient for transmission of the LPS signal are still unknown, raising the possibility that bacteria may move from leukocyte to leukocyte, thus delivering signals to many cells. It is also possible that a few activated host cells can in turn signal and recruit other cells. Thus far, we have not been able to detect signalling activity produced by leukocytes in suspensions of bacteria and PMN or 1% blood. However, such signals may be short-lived or lost under our experimental conditions.

The demonstration that whole bacteria not only deliver potent signals to host cells without extensive shedding of envelope constituents but also continue to signal long after purified LPS is inactivated may have important implications for our thinking about therapeutic intervention in life-threatening clinical infections caused by gram-negative bacteria. A common view is that once destructive responses to LPS have been initiated, the downward cascade cannot be arrested. However, if unlike purified LPS, live or killed bacteria continue to elicit host responses, a greater opportunity may exist for interfering with protracted signalling by administering blocking agents, including BPI (7, 24).

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