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Neutrophil Activation by Bacterial Lipoprotein Versus Lipopolysaccharide: Differential Requirements for Serum and CD14

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Neutrophil activation plays an important role in the inflammatory response to Gram-negative bacterial infections. LPS has been shown to be a major mediator of neutrophil activation which is accompanied by an early down-regulation of L-selectin and up-regulation of CD11b/CD18. In this study, we investigated whether lipoprotein (LP), the most abundant protein in the outer membrane of bacteria from the family Enterobacteriaceae, can activate neutrophils and whether this activation is mediated by mechanisms that differ from those used by LPS or Escherichia coli diphosphoryl lipid A (EcDPLA). Neutrophil activation was assessed by measuring down-regulation of L-selectin and up-regulation of CD11b/CD18. When comparing molar concentrations of LP vs EcDPLA, LP was more potent (four times) at activating neutrophils. In contrast to LPS/EcDPLA, LP activation of neutrophils was serum independent. However, LP activation of neutrophils was enhanced by the addition of soluble CD14 and/or LPS-binding protein. In the presence of serum, LP activation of neutrophils was inhibited by different mAbs to CD14. This inhibition was significantly reduced or absent when performed in the absence of serum. Diphosphoryl lipid A from Rhodobacter spheroides (RaDPLA) completely inhibited LPS/EcDPLA activation of neutrophils but only slightly inhibited LP activation of neutrophils. These results suggest that LP activation of human neutrophils is mediated by a mechanism that is different from LPS activation and that LP is a potentially important component in the development of diseases caused by Gram-negative bacteria of the family Enterobacteriaceae.

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N neutrophils have been shown to be important in host defense to both Gram-positive and Gram-negative bacterial infections. Neutrophil activation plays an important role in the inflammatory response and involves a number of distinct functions that result not only in cytokine production but also in neutrophil migration into infected tissues. For Gram-negative bacteria, LPS (endotoxin) has been shown to be a major mediator of neutrophil activation (1–3). LPS-induced neutrophil activation is accompanied by an early down-regulation of L-selectin and up-regulation of CD11b/CD18 on the cell surface (4–8). L-selectin and CD11b/CD18 are important molecules because they mediate the adhesion of neutrophils to the vascular endothelium. This adhesive response is thought to underlie the movement of neutrophils from the blood into tissues that accompany the early stages of an inflammatory response (9). Furthermore, there is increasing evidence suggesting that integrin-mediated signaling in leukocytes plays a role in the induction of proinflammatory cytokine production (10).

L-selectin is a transmembrane, constitutive monomeric protein on neutrophils. It is cleaved and shed quickly after LPS stimulation in a dose-dependent manner (11–13). CD11b/CD18 (also called MAC-1 or CR3) is a transmembrane, constitutive dimeric glycoprotein on neutrophils. It is a member of the β2 integrin family of proteins formed by the noncovalent association of one α and one β subunit. It is quickly up-regulated by LP in a dose-dependent manner through the mobilization of intracellular granules containing CD11b/CD18 (12).

Only recently have major advances been made in identifying the membrane components and intracellular signaling pathways responsible for LPS-induced cell activation (14). CD14 and LPS-binding protein (LBP) are two proteins important in LPS activation of different cell populations, including neutrophils, macrophages, and endothelial cells (14–18). LBP is an acute phase protein that is present in plasma (19). LBP binds LPS and appears to function by facilitating and accelerating the binding of LPS to membrane CD14 (20). However, LBP can also transfer LPS to high-density lipoprotein, which can result in significant inhibition of cellular responses to LPS (21, 22). Membrane-bound CD14 is a glycosylphosphatidylinositol-anchored protein that has no transmembrane and intracellular domains (23). Activation of neutrophils and macrophages by concentrations of LPS considered physiological (<100 ng/ml) is dependent on the presence of LBP and can be blocked by Ab to CD14 (15). Under serum-free conditions, LPS (<100 ng/ml) fails to activate neutrophils or macrophages because of the absence of LBP (20).

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3 Abbreviations used in this paper: LBP, LPS-binding protein; TLR, Toll-like receptor; LP, lipoprotein; RaDPLA, Rhodobacter spheroides diphosphoryl lipid A; EcDPLA, Escherichia coli diphosphoryl lipid A; sCD14, soluble CD14; HSA, human serum albumin; MFI, mean fluorescence intensity.
There is now strong evidence that LPS-bound CD14 interacts with an as yet unidentified protein(s) that induces a signal transduction event across the plasma membrane, and that LPS binding to CD14 and the subsequent activation are independent processes (24–26). Recently, defective signaling in LPS non-responsive mice was shown to be due to a mutation in the Toll-like receptor (TLR)-4 gene (Tlr4) (27) and TLR4-deficient mice were shown to be hyporesponsive to LPS (28). Toll-like receptor-2 has also been proposed to play a role in LPS activation of different cell populations (29, 30). However, recently, Heine et al. (31) have shown that expression of TLR2 is sufficient but not essential for mammalian responses to LPS. In this regard, Vogel et al. (32) have shown that other receptors besides TLR4 can be involved in LPS signaling of murine macrophages. Thus, the exact role of these proteins in LPS activation is still unclear.

Although LPS has been clearly documented to play an important role in the activation of neutrophils by Gram-negative bacteria, little is currently known about the function of other bacterial-derived components in neutrophil activation. A number of outer membrane proteins contained in Gram-negative bacteria from Enterobacteriaceae have been shown to activate different immune functions. Lipoprotein (LP) is one of these proteins and is the most abundant protein in the outer membrane of these bacteria (33). In Escherichia coli and closely related bacteria, about 10^5 LP molecules are either covalently attached to murein or free in the outer membrane (33). LP or synthetic lipopeptide analogues of LP have been shown to activate mouse B cells and mouse and human macrophages (34–38). We have shown that LP is important in the induction and pathogenesis of septic shock. LP was shown to induce in vitro production of TNF-α and IL-6 by mouse and human macrophages (39, 40) and to induce lethal shock and in vivo production of TNF-α and IL-6 in LPS-responsive and -nonresponsive mice (41). More important, LP was shown to act synergistically with LPS to induce lethal shock and pro-inflammatory cytokine production (41). Recently, we have shown that LP is released by growing or lysed bacteria and that this released LP may play an important role in the pathogenesis associated with Gram-negative bacterial infections (42). However, the potential role of LP in bacterial activation of human neutrophils has not been fully explored, and the possibility that LP can activate neutrophils via receptors that are different from those used by LPS activation has not been investigated. In this report, we show that LP can activate neutrophils, resulting in the up-regulation of CD11b/CD18 and the down-regulation of L-selectin. Neutrophil activation by LP was only partially inhibited by Rhodobacter spheroides diphosphoryl lipid A (RsDPLA), which completely inhibited LPS activation, and LP activation of neutrophils can take place in the absence of serum. In the presence of serum, LP activation is mediated via CD14 but is CD14 independent when activation occurs in the absence of serum. These results have potentially important implications for furthering our understanding of the role of bacterial components in neutrophil activation and bacterial pathogenesis and suggest that LP may play an important role in these processes.

Materials and Methods

Reagents

E. coli K12 LPS (LPS) was extracted from aqueous suspensions of overnight cultures with 45% phenol at 70°C as we have described previously (39). Bacterial LP was purified from E. coli, Yersinia enterocolitica WA (0:8), Salmonella typhimurium (TML), and Shigella flexneri (SA 100) as described previously (39). LP purified from these different bacteria contained <25 μg of LPS/mg of protein and was shown to be pure by SDS-PAGE and by HPLC. Diphosphoryl lipid A from E. coli K12 (EcDPLA) (molecular mass, 1900 Da) was purchased from List Laboratories (Campbell, CA), and prepared according to the manufacturer’s specifications. RsDPLA was purified as described previously (43). LPS, LP, EcDPLA, and RsDPLA were dispersed by sonication for 3 min on ice before use. Three different hybridsomas that produce Abs specific for human CD14 were cultured from the American Type Culture Collection (Manassas, VA). The anti-CD14 hybridomas were 3C10, 60bca, and 26ic. The Abs specific for human CD14 and isotype control Abs were purified from in vitro culture supernatants using protein G chromatography. Dr. H. Lichtenstein (Amen, CA) provided purified human LBP and human soluble CD14 (sCD14).

All other chemicals used in this study were purchased from Sigma (St. Louis, MO). All plastic materials were sterile and all glass materials were sterilized and baked overnight at 263°C to destroy endotoxin.

Neutrophil purification and activation

Neutrophil purification and culture were performed following the procedure described by others (8, 12, 44) with slight modifications. Briefly, venous blood was collected from normal human volunteers in Vacutainer (Becton Dickinson, San Jose, CA) collection tubes containing 15% K,EDTA solution. The polymorphonuclear leukocytes (PMNL) were isolated by centrifugation of the whole blood in tubes containing neutrophil-isolating media (Cardinal Associates, Santa Fe, NM) or mono-poly resolving medium (ICN Pharmaceuticals, Aurora, OH) for 35 min, at 400 × g, at 15°C. Purified PMNL were washed twice with ice-cold HEPES buffer (30 mM HEPES, 110 mM NaCl, 10 mM KCl, and 10 mM glucose) and resuspended in HEPES buffer supplemented with calcium (1.5 mM CaCl₂), magnesium (1 mM MgCl₂), and FCS (5% heat inactivated). Cell purification using the procedure described above resulted in PMNL preparations that were 98% PMNL, of which 99% were neutrophils and 1% mononuclear leukocytes, as shown by Wright’s staining of cytosin preparations. Cell viability of these cells was >97%, as determined by trypan blue exclusion. Neutrophils, as isolated above, were kept on ice until used as described below.

The effect of LP vs LPS or EcDPLA on neutrophil surface expression of L-selectin and CD11b/CD18 was determined following the procedure described by others (8, 12, 44), with modifications. Briefly, neutrophils (1 × 10⁶/ml) in HEPES buffer supplemented with calcium, magnesium, and FCS were incubated with various amounts of LP, LPS, EcDPLA, or RsDPLA (10 μM) in polystyrene, sterile, round-bottom tubes in a water bath at 37°C for 30 min. Surface L-selectin and CD11b/CD18 were determined by flow cytometry as described below.

In some experiments, LP and LPS were assessed for their ability to activate neutrophils in the presence and absence of serum. In the serum-free condition, the FCS was replaced by 300 μg/ml human serum albumin (HSA) (Albunarc; Baxter, McGraw Park, IL). Effect of purified LBP and/or sCD14 on neutrophil activation by LP vs EcDPLA or LPS

Purified human neutrophils (1 × 10⁶/ml) in HEPES buffer containing calcium, magnesium, HSA (300 μg/ml), LBP (10 ng/ml), and/or sCD14 (1 μg/ml) and various doses of LP, EcDPLA, or LPS, were positive control for these experiments consisted of purified neutrophils incubated in HEPES buffer supplemented with calcium, magnesium, 5% FCS, and various doses of LP, EcDPLA, or LPS. The cells were incubated for 30 min in a water bath at 37°C and surface L-selectin and CD11b/CD18 were determined by flow cytometry.

Treatment of neutrophils with mAbs to CD14

Purified human neutrophils (1 × 10⁶/ml) in HEPES buffer containing calcium and magnesium were pretreated for 1 h at 4°C with 10 μg/ml of either mAb to human CD14 (3C10, 60bca, or 26ic) or an isotype control Ab. In some experiments, the effect of mAb to human CD14 on LP-induced modulation of neutrophil surface markers was determined both in the presence and absence of FCS. In these experiments, HSA (300 μg/ml) was present in cultures containing no serum. Following pretreatment with Abs, various amounts of LP or LPS were added to each tube and the cells were incubated for 30 min in a water bath at 37°C. The cells were then assessed for surface L-selectin and CD11b/CD18 by flow cytometry as described below. The results of all of the above experiments are expressed as percent inhibition of L-selectin down-regulation and as percent inhibition of CD11b/CD18 up-regulation.

Effect of RsDPLA on neutrophil activation induced by LP vs EcDPLA or LPS

Purified human neutrophils (1 × 10⁶/ml) in HEPES buffer containing calcium, magnesium, and 5% FCS were incubated with and without RsDPLA (100 ng/ml) for 1 h at 37°C. Then various doses of LP, LPS, or EcDPLA were dispersed by sonication for 3 min on ice before use. Three different hybridsomas that produce Abs specific for human CD14 were cultured from the American Type Culture Collection (Manassas, VA). The anti-CD14 hybridomas were 3C10, 60bca, and 26ic. The Abs specific for human CD14 and isotype control Abs were purified from in vitro culture supernatants using protein G chromatography. Dr. H. Lichtenstein (Amen, CA) provided purified human LBP and human soluble CD14 (sCD14).
were added to the cultures and the cells were incubated for 30 min at 37°C. Surface L-selectin and CD11b/CD18 were determined by flow cytometry.

**Flow cytometry analysis of surface L-selectin and CD11b/CD18**

Surface L-selectin and CD11b/CD18 were determined by direct double staining using FITC-conjugated anti-human L-selectin (PharMingen, San Diego, CA) and PE-conjugated anti-human CD11b/CD18 (Becton Dickinson). Neutrophils (1 x 10⁶/ml), resuspended in cold PBS (0.1 M phosphate buffer, 0.85% NaCl, 1% FCS (pH 7.4)), were incubated with both fluorochrome-conjugated Abs for 30 min at 4°C. The cells were washed once with cold PBS, fixed with 2% p-formaldehyde, and analyzed using a Becton Dickinson FACScan instrument at the Flow Cytometry Core Facility of the Department of Microbiology and Immunology (University of Texas Medical Branch). The results are reported as percentage of maximal L-selectin expression and percentage of maximal CD11b/CD18 expression. L-selectin maximal MFI is the value corresponding to cells treated with 100 ng/ml of LPS. CD11b/CD18 maximal MFI is the value corresponding to control cells not stimulated with either LP or LPS. These results are expressed as percentage of maximal L-selectin expression and percentage of maximal CD11b/CD18 expression.

**Results**

**LP activation of neutrophils**

In initial experiments, we investigated whether LP could regulate neutrophil expression of L-selectin and/or CD11b/CD18. For these experiments, we compared *E. coli* LP vs LPS vs EcDPLA for their ability to activate neutrophils. Data presented in Figs. 1-3 show that LP can down-regulate L-selectin and up-regulate CD11b/CD18 expression in neutrophils. LP-induced neutrophil activation was comparable to that of LPS. LP was also compared on a molar basis to EcDPLA. As shown in Fig. 2, LP was at least four times more potent than EcDPLA at inducing neutrophil activation. Optimal neutrophil activation by LP, LPS, or EcDPLA took place at around 20 min after exposure (Fig. 3). Neutrophil activation was not unique to *E. coli* LP, since LP purified from other members of

LP down-regulates L-selectin (A) and up-regulates CD11b/CD18 (B) on human neutrophils. Purified human neutrophils were treated with various amounts of LP (■) or LPS (□), and the MFI values corresponding to L-selectin and CD11b/CD18 were determined. The results are expressed as percentage of maximal L-selectin expression (A) and percentage of maximal CD11b/CD18 expression (B) vs concentration of LP or LPS in ng/ml. This is a representative example of 10 independent experiments. L-selectin maximal MFI = 123 ± 11. CD11b/CD18 maximal MFI = 1033 ± 22.

- **FIGURE 1.**

Comparison of the effect of equimolar doses of LP vs EcDPLA on the down-regulation of L-selectin (A) and the up-regulation of CD11b/CD18 (B). Purified human neutrophils were incubated with the same molar concentrations of LP (■) or EcDPLA (□), and the MFI values corresponding to L-selectin and CD11b/CD18 were determined. The results are expressed as percentage of maximal L-selectin expression (A) and as percentage of maximal CD11b/CD18 expression (B) vs molar concentration of LP and EcDPLA. This is a representative example of four independent experiments. L-selectin maximal MFI = 48 ± 3. CD11b/CD18 maximal MFI = 602 ± 112.
Enterobacteriaceae (Y. enterocolitica, S. typhimurium, and S. flexneri) had similar activity (Fig. 4).

Serum requirement for LP activation of neutrophils

To investigate whether LP activation of neutrophils was dependent on serum, as has been reported for LPS, we performed a series of experiments comparing the ability of LP vs LPS vs EcDPLA to activate neutrophils in the presence or absence of serum. Neutrophils were exposed to varying concentrations of LP vs LPS vs EcDPLA in the presence or absence of FCS and assessed for changes in L-selectin and CD11b/CD18. As previously reported by others (14), LPS or EcDPLA failed to activate neutrophils under serum-free conditions (Fig. 5, C and D). In contrast, LP activation of neutrophils was not affected by the absence of serum (Fig. 5, A and B). This result was observed using a wide range of LP concentrations.

Role of LBP and CD14 in LP-induced neutrophil activation

LBP and sCD14 have been shown to play an important role in LPS activation of different cell populations, including neutrophils (14–18, 45). Since LP could activate neutrophils in the absence of serum, we investigated whether LP-induced activation of neutrophils was affected by LBP and/or sCD14. To investigate this possibility, we compared LP vs EcDPLA for their ability to activate neutrophils in the presence of LBP and/or sCD14 under serum-free conditions. Data from a representative experiment are presented in Fig. 6. Under serum-free conditions, both LBP and sCD14 enhanced neutrophil activation by LP. An even more dramatic enhancement was observed when both LBP and sCD14 were present during the assays. As previously reported, EcDPLA was completely inactive when no serum or LBP or sCD14 was present, but LBP, sCD14, and a combination of both (LBP/sCD14) resulted in significant neutrophil activation (Fig. 6, C and D). Identical results were obtained in experiments using LPS (data not presented). These results show that LP activation can be enhanced by LBP and/or sCD14, even though LP can activate neutrophils in the absence of serum, LBP, or sCD14. These results suggest that LP activation of neutrophils could be mediated by different mechanisms depending upon whether activation occurred in the presence of serum or in tissues where serum would be limited.

LPS activation of neutrophils has been shown to be dependent on LPS interaction with membrane CD14 (14–18, 45). To investigate whether LP activation of neutrophils was also dependent on...
membrane CD14, we performed a series of experiments comparing the ability of LP vs LPS to activate neutrophils that had been pretreated with different mAbs specific for different CD14 epitopes. For these experiments, we used the following mAbs: 3C10, 60bca, and 26ic. 3C10 and 60bca have both been shown to markedly inhibit LPS-mediated effects on CD14-positive cells (45–47), whereas 26ic has been shown to bind to CD14 but does not inhibit LPS-induced cell activation (14). We assessed neutrophils using LP vs LPS at 32 and at 128 ng/ml and in the presence of serum with or without 10 ng/ml of each of the different mAbs or the corresponding isotype control Ab. Data from a representative experiment are presented in Fig. 7. In the presence of serum, both 60bca and 3C10 almost completely inhibited neutrophil activation by LPS at both 32 and 128 ng/ml and in the presence of serum with or without 10 μg/ml of each of the different mAbs or the corresponding isotype control Ab. Data from a representative experiment are presented in Fig. 7. In the presence of serum, both 60bca and 3C10 almost completely inhibited neutrophil activation by LPS at both 32 and 128 ng/ml. For LP activation of neutrophils, 60bca was also inhibitory when LP was assessed at 32 ng/ml. However, when LP was assessed at 128 ng/ml, 60bca was dramatically less efficient at inhibiting LP activation of neutrophils when compared with LP activation at 128 ng/ml. Interestingly, 3C10 had significantly less inhibitory activity for LP when compared with LPS. In fact, when LP was assessed at 128 ng/ml, 3C10 had only marginal inhibitory activity (L-selectin = 5 ± 1% inhibition; CD11b/CD18 = 11 ± 2% inhibition), whereas LPS activation (using 128 ng/ml) was still significantly inhibited by 3C10 (L-selectin = 86 ± 10% inhibition; CD11b/CD18 = 88 ± 13% inhibition). Only very low levels of inhibitory activity were observed when 26ic was assessed for LPS or LP activation of neutrophils. Collectively, the above results suggest that LP activation of neutrophils is CD14 dependent when performed in the presence of serum, and that LP and LPS may interact with CD14 differently as evidenced by the differential inhibition mediated by 3C10 vs 60bca.

Since LP could activate neutrophils in the absence of serum, we next investigated whether the different anti-CD14 mAbs described above could alter neutrophil activation by LP under serum-free conditions. As seen in Fig. 8, significantly less inhibition was observed when these Abs were assessed using serum-free conditions. This was most dramatic when LP was assessed at 128 ng/ml. At this concentration, and in the absence of serum, 3C10 had almost no inhibitory activity against LP activation of neutrophil expression of CD11b/CD18 or L-selectin. These results suggest that LP activation of neutrophils is CD14 independent when activation takes place in the absence of serum.

RsDPLA does not inhibit LP activation of neutrophils

A number of studies have shown that RsDPLA can inhibit LPS activation of different cell populations (43, 45, 48, 49). We next investigated whether RsDPLA could also inhibit LP activation of neutrophils. Neutrophils were pretreated with either medium or medium plus RsDPLA (100 ng/ml) for 1 h and then assessed for their ability to be activated by varying concentrations of LP vs EcDPLA. As seen in Fig. 9, RsDPLA completely inhibited EcDPLA activation of neutrophils. Identical results were obtained when using LPS (data not presented). In contrast, RsDPLA had significantly less of an effect on LP activation of neutrophils.

FIGURE 5. LP-induced down-regulation of L-selectin and up-regulation of CD11b/CD18 is serum independent. Purified human neutrophils were treated with various doses of LP or LPS in medium with or without FCS (5%). A and B, The MFI values corresponding to LP-induced down-regulation of L-selectin (A) and up-regulation of CD11b/CD18 (B) with (■) and without (□) FCS. C and D, The MFI values corresponding to LPS-induced down-regulation of L-selectin (C) and up-regulation of CD11b/CD18 (D) with (■) and without (□) FCS. The results are presented as percentage of maximal L-selectin expression and percentage of maximal CD11b/CD18 expression vs LP and LPS concentration in ng/ml.
FIGURE 6. Purified LBP and/or sCD14 enhance LP-induced down-regulation of L-selectin and up-regulation of CD11b/CD18. Purified human neutrophils were incubated with the same molar doses of LP or EcDPLA in the following conditions: 5% FCS (●), no FCS (★), LBP (◆), sCD14 (▲), and LBP and sCD14 (■). The results are presented as percentage of maximal L-selectin expression and percentage of maximal CD11b/CD18 expression. A and B, Results using LP. For LP, the results in the presence and absence of serum were identical, and only the data obtained in the absence of serum is shown. C and D, Results obtained using EcDPLA. This is a representative example of three independent experiments. L-selectin maximal MFI = 48 ± 6. CD11b/CD18 maximal MFI = 432 ± 95.

FIGURE 7. LP- and LPS-induced modulation of cell surface markers are inhibited differently by anti-CD14 mAbs. Purified human neutrophils were incubated with 10 μg/ml of anti-CD14 mAbs 3C10, 60bca, and 26ic or the corresponding isotype controls for 1 h before LP (●) or LPS (▲) was added to the cultures at 32 and 128 ng/ml. The results are presented as average percent inhibition of L-selectin down-regulation ± SD (A and B) and average percent inhibition of CD11b/CD18 up-regulation ± SD (C and D; n = 3). *, Those LP values that are statistically different from the corresponding values for LPS (Student’s t test, p < 0.01).
FIGURE 8. The anti-CD14 mAb-mediated inhibition of LP-induced modulation of neutrophil surface markers is significantly reduced in the absence of serum. Purified human neutrophils were incubated with 10 μg/ml of anti-CD14 mAbs 3C10, 60bca, and 26ic or the corresponding isotype controls for 1 h, both in the presence (●) and absence of FCS (□). LP was then added to the cultures at the final concentration of 32 or 128 ng/ml. The MFI values corresponding to L-selectin and CD11b/CD18 were determined using flow cytometry. The results are expressed as average percent inhibition of L-selectin down-regulation ± SD (A and B) and average percent inhibition of CD11b/CD18 up-regulation ± SD (C and D; n = 3). *, The value for LP determined in the presence of serum is statistically different from the corresponding value determined in the absence of serum (Student’s t test, p < 0.01).

FIGURE 9. RsDPLA partially inhibits LP-induced modulation of surface L-selectin and CD11b/CD18. Purified human neutrophils were incubated with (□) or without (●) RsDPLA (100 ng/ml) for 1 h. After this preincubation, LP (A and B) or EcDPLA (C and D) was added to the cultures at equimolar doses and surface L-selectin and CD11b/CD18 were determined by flow cytometry. The results are expressed as percentage of maximal L-selectin expression (A and C) and percentage of maximal CD11b/CD18 expression (B and D). This is a representative example of three independent experiments. L-selectin maximal MFI = 75 ± 8. CD11b/CD18 maximal MFI = 789 ± 95.
These results, again, suggest that LP and EcDPLA/LPS use different mechanism(s) and/or receptors for neutrophil activation.

**Discussion**

In this report, we present evidence that LP may play an important role in the inflammation associated with infections caused by bacteria of the *Enterobacteriaceae* family. The following observations were made in this study: 1) LP obtained from different members of the *Enterobacteriaceae* family can activate human neutrophils. 2) LP activation can take place in the absence of serum. 3) In the presence of serum, Abs to CD14 can inhibit LP-induced activation, but this inhibition, depending on the Ab, disappears as the concentration of LP is increased. 4) In the absence of serum, Abs to CD14 have little to no inhibitory activity for LP-induced neutrophil activation. 5) LBP and sCD14 can enhance LP-induced neutrophil activation. 6) RsDPLA fails to significantly inhibit LP-induced neutrophil activation but can completely inhibit LPS-induced neutrophil activation.

Neutrophil activation by LP could be achieved using 2–10 ng/ml, which was comparable to the concentrations of LPS necessary for neutrophil activation. However, in most experiments, optimal neutrophil activation could be achieved using concentrations of LP that were lower than concentrations required by LPS for neutrophil activation. This was confirmed by experiments that compared on a molar basis the ability of LP vs EcDPLA to activate neutrophils. It should be pointed out that in some experiments neutrophils obtained from some individuals responded better to LPS than LP (data not presented). Although only suggestive, these results indicate that LP and LPS may mediate neutrophil activation via different mechanisms that could be associated with differential expression of surface receptors by different individuals. The fact that nanogram levels of LP could activate neutrophils is significant in light of our previous work. Using a dot blot assay, we showed that growing bacteria released significant levels of both LP and LPS (42). During mid-logarithmic growth, &lt;1–1.5 &mu;g of LP/ml was detected in culture supernatants from different bacteria of the *Enterobacteriaceae*. Thus, the possibility exists that LP released in vivo by growing or lysed bacteria could contribute or induce an inflammatory response.

A striking difference between LPS and LP for activating neutrophils was the fact that LP could accomplish this in the complete absence of serum. This was observed using a wide range of concentrations of LP. The capacity of LP to activate neutrophils under serum-free conditions could be important for inducing an in vivo inflammatory response in tissues where serum is limited. Monocyte and neutrophil activation by lipoproteins from *Treponema pallidum* and *Borrelia burgdorferi* or by lipopeptides corresponding to the N termini of these proteins have also been shown to be serum independent (50–52). In fact, during the last couple of years, a significant amount of information has accumulated about the role of spirochetal lipoproteins in the inflammation associated with syphilis and Lyme disease and how these lipoproteins activate different cell populations differently from LPS (50–60). Additionally, LP from Gram-negative bacteria and synthetic lipopeptide analogues of LP have been shown by a number of laboratories to be capable of activating different cell populations (61–68). Whether LP produced by bacteria from the *Enterobacteriaceae* family and lipoproteins produced by spirochetes activate cell populations via similar or different receptors and/or signaling pathways is unclear. In this regard, we found that LP activation of neutrophils could be significantly enhanced by the presence of LBP and/or sCD14. In contrast, activation of human monocytes by spirochetal lipoproteins was shown not to be enhanced or changed in the presence of LBP and/or sCD14 (51, 52). This appears to be a clear difference between LP and spirochetal lipoproteins but could also reflect different requirements for different cell populations since we were assessing neutrophils and spirochetal lipoproteins were assessed on human monocyte cell lines. In fact, spirochetal lipoproteins were shown to activate HUVECs, and this activation was enhanced by serum or sCD14 (53). It should also be noted that Sellati et al. (52) found suggestive evidence that there could be differences in the biological activity of spirochetal lipoproteins vs their synthetic analogues (lipopeptides). It is possible that LP from *Enterobacteriaceae* could act differently from lipoproteins produced by spirochetes. However, the biological activities of both LP and lipoproteins produced by spirochetes are dependent on the presence of the fatty acids at the N terminus of these proteins. This has been demonstrated by a number of laboratories using lipopeptides and LP that had its lipid moiety removed or altered (51, 57, 65, 66, 69, 70). Thus, the lipid portion of LP is also probably necessary for LP activation of neutrophils.

A number of different bacterial products, other than LPS, have been shown to activate different cell populations via a CD14-dependent pathway (71–74). Monocyte activation by spirochetal lipoprotein was also shown to be inhibited by mAbs specific for CD14 (51, 52). However, LPS and spirochetal lipoprotein differed in that signaling by lipoprotein proceeded via distinct transducing elements downstream of CD14 (52). We also found that LP activation of neutrophils could be significantly inhibited by 3C10 and 60bca mAbs. Interestingly, this inhibition was much less dramatic for 3C10 when compared with 60bca. In fact, 3C10 had little inhibitory activity when LP was assessed at 128 ng/ml even though LPS at this concentration was still almost completely inhibited by this Ab. These results suggest that LP may interact with CD14 differently from LPS. The fact that LP could activate neutrophils in the absence of serum suggested that a CD14-independent mechanism could also be involved in LP activation. In fact, mAbs to CD14 (3C10 and 60bca) had significantly less or no inhibitory activity when assessed under serum-free conditions. These results suggest that LP can activate neutrophils via both CD14-dependent and -independent mechanisms. This is not unique to LP since a CD14-independent stimulatory pathway also becomes apparent at progressively higher concentrations of LPS (75) and spirochetal lipoproteins (51). However, our results suggest that LP can activate via a CD14-independent pathway at significantly lower concentrations when compared with LPS. Whether LP mediates cell activation via signaling pathways different from spirochetal lipoproteins or LPS is currently under investigation. Additionally, whether LP mediates cellular signaling via TLR2 and/or TLR4 is unclear. Since LP can activate macrophages from LPS-nonresponsive mice (C3H/HeJ) (41), TLR4 does not appear to be necessary for LP activation of cells. This is clearly a difference between LP and LPS activation and suggests that different membrane transducers may be used by these bacterial products. In fact, we have shown that LP and LPS act synergistically in inducing cytokine production by mouse macrophages and for inducing lethal shock in mice (41). However, humans and mice may use different signaling and/or receptor pathways with regard to LP and LPS signaling. For example, we have not been able to show any synergistic interaction between LP and LPS for human neutrophil activation (data not presented).

LPS activation of different cell populations has been shown to be blocked by the naturally occurring antagonist RsDPLA. When present at low concentrations, RsDPLA acts in a manner consistent with competitive inhibition of LPS activation, whereas at higher concentrations, it inhibits certain LPS responses noncompetitively and synergizes with LPS for other responses (44, 45, 48, 49, 76–
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78. RsDPLA had very different effects on LP vs LPS activation of neutrophils. Although LPS was completely inhibited by RsDPLA, LP activation of neutrophils was only slightly inhibited when optimal concentrations of LP were studied. These results further strengthen our conclusion that LP activates neutrophils differently from LPS.

In summary, our results strongly suggest that LP-induced activation of human neutrophils is mediated by a mechanism(s) that is different from LPS-activated induction and that LP is a potentially important component in the development of diseases caused by Gram-negative bacteria of the Enterobacteriaceae.

References


