# DEACYLATED LIPOPOLYSACCHARIDE INHIBITS NEUTROPHIL ADHERENCE TO ENDOTHELIUM INDUCED BY LIPOPOLYSACCHARIDE IN VITRO

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A role for endothelial cells in the inflammatory response to Gram-negative LPS is suggested by several lines of evidence: low doses of LPS induce expression of a cell surface factor(s) that increases the adherence of neutrophils to the endothelial cell surface (1, 2), release of IL-1 (3, 4), release of an inhibitor of tissue plasminogen activator (5, 6), and the expression of tissue factor (7). These changes may contribute to the perivascular emigration of neutrophils and local thrombosis that occur at sites of Gram-negative bacterial tissue invasion.

A standard experimental model for LPS tissue toxicity is the dermal Shwartzman reaction. Here an intradermal (preparative) injection of LPS provokes an inflammatory reaction that is characterized by leukocyte adherence to endothelium and then emigration of leukocytes from the vascular lumen into the surrounding tissue space. When an intravenous injection of LPS is given 20–24 h later, hemorrhage and necrosis develop at the intradermal injection site (8). The mechanism by which LPS initiates neutrophil adherence and migration in this reaction is uncertain. Recent studies (9) have shown, however, that certain structural requirements are necessary for lipid A, the toxic moiety of LPS, to produce the dermal Shwartzman reaction: a glucosamine disaccharide, phosphates at positions 1 and 4, and at least one acyloxyacyl group. For example, monophosphoryl lipid A, which lacks phosphate at carbon 1, does not prepare the skin for the dermal Shwartzman reaction (9).

It was recently reported (10) that enzymatic removal of the nonhydroxylated fatty acids that are attached by acyloxyacyl linkages to the ester- and amide-linked long chain fatty acids of the lipid A region of LPS reduces the activity of LPS in the dermal Shwartzman reaction by a factor of $\geq$100. Since adherence of neutrophils to endothelium may contribute to the pathogenesis of the dermal Shwartzman reaction, we studied the effects of LPS, enzymatically deacylated LPS, lipid X (2,3-diacylglucosamine-1-phosphate [11], a nontoxic precursor of

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lipid A), and monophosphoryl lipid A on neutrophil adherence to cultured human umbilical vein endothelium (HUVE).[1] We found that pretreatment of HUVE with LPS markedly increased neutrophil adherence, whereas HUVE pretreated with enzymatically deacylated LPS, lipid X, or monophosphoryl lipid A failed to acquire adhesiveness for neutrophils. Of additional interest was our finding that deacylated LPS, but not lipid X or monophosphoryl lipid A, inhibited neutrophil adherence to HUVE induced by pretreatment of HUVE with LPS.

## Materials and Methods

*Reagents.* Recombinant human tumor necrosis factor $\alpha$ (rhTNF-$\alpha$) was a gift of Genentech, Inc., South San Francisco, CA. The preparation, assayed (by the supplier) for cytolytic activity on actinomycin D–treated L929 mouse fibroblast cells, contained $5.02 \times 10^7$ U/ml. Recombinant human IL-1 (rhIL-1) ($10^9$ U/ml) was purchased from Genzyme (Boston, MA). The crystalline ditris salt of lipid X was provided by Dr. Ingolf Macher, Sandoz Forschungsinstitut, Vienna, Austria (12). The crystalline material was dissolved in chloroform/methanol 2:1 (vol/vol), aliquoted, dried under $N_2$, and stored at −70°C. Immediately before use, an aliquot of lipid X was dissolved in RPMI 1640 (M. A. Bioproducts, Walkersville, MD) by waterbath sonication. Monophosphoryl lipid A from *Salmonella typhimurium* was purchased from Ribi ImmunoChem Research, Inc. (Hamilton, MT). It was suspended in PBS without $Mg^{2+}$ or $Ca^{2+}$; (Grand Island Biological Co., Grand Island, NY) and sonicated for 10–20 sec after warming to 65°C. *Escherichia coli* (055:B5) LPS, extracted by phenol-water (Westphal), was obtained from Sigma Chemical Co., St. Louis, MO.

*Preparation of Deacylated LPS.* Biosynthetically radiolabeled Rc LPS were prepared from *S. typhimurium* PRX20 as described (10, 13). The LPS contained 107,000 dpm of $^3$H-labeled fatty acid and 3,750 dpm of $^{14}$C-labeled glucosamine per microgram. Enzymatic deacylation was performed using acyloxyacyl hydrolase that was partially purified (~1,500-fold enrichment in specific activity) from HL-60 cells. To produce maximal enzymatic deacylation, LPS (20 $\mu$g) was incubated with enzyme for 72 h at 37°C in a reaction mixture that contained BSA (1 mg/ml), 5 mM $CaCl_2$, 0.5% (vol/vol) Triton X-100, and 20 mM Tris-citrate, pH 4.8. The reaction was stopped by precipitating the LPS and BSA with three volumes of 95% ethanol. The precipitates were washed with 80% ethanol, resuspended in pyrogen-free saline, and frozen in aliquots at −70°C. The percent deacylation (percent loss of $^3$H radioactivity from the LPS) was calculated by counting the radioactivity in the precipitate and in the ethanol supernatant; analysis of the deacylated LPS and the ethanol-soluble fatty acids by thin-layer chromatography indicated that the maximally deacylated LPS (32% loss of $^3$H radioactivity) retained ~12% of the original $^3$H-nonhydroxylated fatty acids and >90% of the $^3$H-3-hydroxytetradecanoate. Graded deacylation was performed by incubating constant amounts of enzyme and LPS for various time intervals, as described (10). Control LPS, incubated in the reaction mixture without enzyme, and the reaction mixture with enzyme, incubated without LPS, were precipitated and stored as described for deacylated LPS.

*Cell Culture.* Endothelial cells were prepared by collagenase treatment of human umbilical veins as previously described (14). Cultures were maintained in endotoxin-free RPMI 1640 supplemented with 20% FCS (Hyclone, Sterile Systems, Logan, UT), heparin (90 $\mu$g/ml; Sigma Chemical Co.), and endothelial cell growth factor (150 $\mu$g/ml) as described by Thornton et al. (15). Endothelial cell growth factor was prepared from bovine hypothalamus according to the method of Maciag et al. (16).

*Neutrophil Isolation and Labeling.* Peripheral blood was obtained by venipuncture from healthy donors. The blood was collected in syringes containing heparin (10 U/ml), and the neutrophils were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway,

[1] *Abbreviations used in this paper:* HUVE, human umbilical vein endothelium; rhIL-1, recombinant human IL-1; rhTNF-$\alpha$, recombinant human tumor necrosis factor $\alpha$.

NJ) gradient centrifugation, 3% dextran sedimentation, and hypotonic saline lysis (17). This procedure resulted in a preparation >95% neutrophils, which exceeded 95% viability of trypan blue dye exclusion. Isolated peripheral blood neutrophils were suspended in PBS at $10^6$ cells/ml and were labeled for 60 min at 37°C with 1 $\mu$Ci/$\mu$l $^{51}$Cr as sodium chromate, 1 mCi/ml, 200–500 Ci/g (New England Nuclear, Boston, MA) (18). After labeling, the cells were washed three times with PBS and suspended at a final concentration of $2 \times 10^6$ cells/ml in RPMI 1640 containing 1% FCS and polymyxin B (10 $\mu$g/ml; Sigma Chemical Co.).

*Adherence Assay.* $^{51}$Cr-labeled neutrophil adherence to HUVE was measured as previously described (1, 19). Primary cultures of HUVE were harvested with 0.05% trypsin and 0.02% EDTA in HBSS (Gibco Laboratories, Grand Island, NY). The cells were then plated in 11-mm diameter wells (Costar Cluster; Costar, Cambridge, MA) at $5 \times 10^4$ cells/well in RPMI 1640 supplemented with 20% FCS. Visually confluent monolayers were formed after overnight incubation. To measure endothelial-dependent neutrophil adherence, HUVE were pretreated with 5% FCS and medium alone (control) or medium containing test reagents for 4 h and then washed with two well-volume exchanges of medium supplemented with 5% FCS. Polymyxin B (10 $\mu$g/ml) was included in the wash medium to inactivate free or surface-bound LPS. $^{51}$Cr-labeled neutrophils were then added (250 $\mu$l/well), and the cells were incubated for 30 min at 37°C. After incubation, HUVE were washed with a single well-volume exchange of medium to remove nonadherent neutrophils. The cell layer and the remaining adherent $^{51}$Cr-labeled neutrophils were solubilized by addition of 1 N $NH_4OH$ and the lysates were counted in a gamma counter (Micromedic Systems, Inc., Horsham, PA).

*B Cell Mitogenicity Assay.* B cell mitogenicity of LPS and deacylated LPS was measured as previously described (10). LPS or deacylated LPS was added to single-cell suspensions of $C_3H$/HeN mouse spleen in 96-well microtiter plates; cultures (0.2 ml) contained 300,000 cells in RPMI 1640 with antibiotics and 5% FCS. After 24 h, methyl-[$^3$H]-thymidine (0.5 $\mu$Ci) was added, and the amount of radioactivity incorporated into the cells in each well was measured 18 h later.

## Results

*Deacylated LPS Lacks Activity.* Pretreatment of HUVE with LPS increased the adherence of subsequently added neutrophils. Neutrophil adherence to HUVE pretreated with LPS (13 ng/ml for 4 h) was 31 ± 2% compared with 4 ± 1% adherence to untreated HUVE (means ± SE of seven experiments). In contrast, pretreatment of HUVE with enzymatically deacylated LPS did not promote the adherence of subsequently added neutrophils. Neutrophil adherence to HUVE pretreated with deacylated LPS (126 ng/ml for 4 h) was 6 ± 2% compared with 8 ± 2% adherence to untreated HUVE (means ± SE of four experiments). Partial removal of the nonhydroxylated fatty acids by graded exposure of LPS to the acyloxyacyl hydrolase produced LPS preparations with intermediate activity. Neutrophil adherence to HUVE pretreated with deacylated LPS decreased with the extent of enzymatic deacylation (Fig. 1). In contrast to the lack of activity of deacylated LPS in promoting neutrophil adherence of HUVE, the B cell mitogenic activity of LPS was only minimally affected by enzymatic deacylation of LPS (Fig. 2).

*Deacylated LPS Inhibits the Activity of LPS.* Coincubation with deacylated LPS reduced neutrophil adherence induced by pretreatment of HUVE with LPS. Complete inhibition was observed when the ratio of deacylated LPS to LPS (wt/wt) was >20-fold (Fig. 3). Deacylated *S. typhimurium* LPS also inhibited *E. coli* LPS. Neutrophil adherence to HUVE pretreated for 4 h with *E. coli* LPS

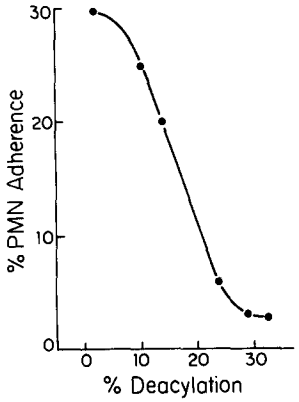


FIGURE 1. Neutrophil adherence to HUVE pretreated with LPS decreases as the extent of LPS deacylation increases. HUVE were pretreated for 4 h with LPS preparations (6.7 ng/ml) that differed in the extent of removal of the acyloxyacyl-linked nonhydroxylated fatty acids. HUVE were then washed twice and $^{51}$Cr-labeled neutrophils were added. Percent neutrophil adherence was determined after a 30-min incubation. Values represent the mean of four replicate wells for each LPS preparation in one representative experiment.

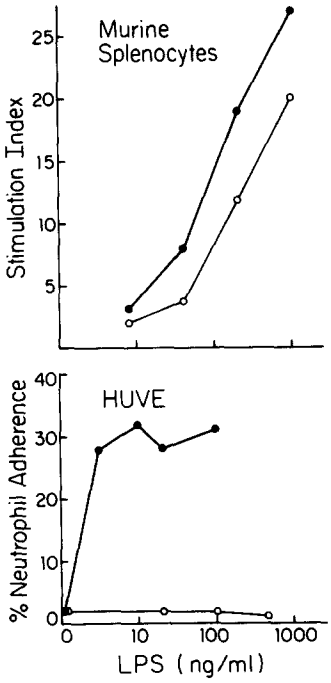


FIGURE 2. Deacylated LPS fails to induce neutrophil adherence to HUVE, but retains activity as a B cell mitogen. (*Top*) Murine spleen cell suspensions were treated for 18 h with medium containing LPS (*closed circles*) or medium containing deacylated LPS (*open circles*) in varying concentrations. [$^3$H]-Thymidine was added and the stimulation index was determined after an 18-h incubation. Values represent the average of four determinations. (*Bottom*) HUVE were pretreated for 4 h with medium containing LPS (*closed circles*) or medium containing deacylated LPS (*open circles*) in varying concentrations. HUVE were then washed twice and $^{51}$Cr-labeled neutrophils were added for a 30-min incubation. Values represent the means of four replicate wells in one representative experiment.

(25 ng/ml) was 31 ± 2%, but was reduced to 3 ± 1% when deacylated *S. typhimurium* LPS (268 ng/ml) was added with the *E. coli* LPS (means ± SD of 4 replicate wells). Maximum inhibition was observed when deacylated LPS was added to HUVE 30 to 60 min before LPS; but addition of deacylated LPS to HUVE as late as 60 min after the addition of LPS significantly reduced subsequent neutrophil adherence (Fig. 4).

Similar to LPS, pretreatment of HUVE with rhTNF-α (1, 19) or rhIL-1 (1, 2, 20) produces an increase in neutrophil adherence to HUVE. Deacylated LPS did not inhibit neutrophil adherence induced by pretreatment of HUVE with either rhTNF-α or rhIL-1 (Table I). Like deacylated LPS, lipid X and monophosphoryl lipid A (21) failed to induce the adherence-promoting activity in

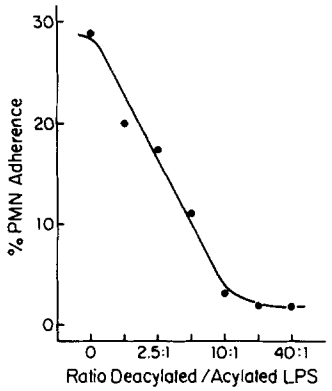


FIGURE 3. Deacylated LPS inhibits neutrophil adherence to HUVE induced by pretreatment of HUVE with LPS. HUVE were pretreated for 30 min with deacylated LPS in varying concentrations. Without washing, LPS (13.4 ng/ml) was then added, and HUVE were incubated for an additional 4 h. HUVE were then washed and $^{51}$Cr-labeled neutrophils were added. Percent neutrophil adherence was determined after a 30-min incubation. Values represent the mean of two replicate wells at each ratio (wt/wt) of deacylated LPS to LPS in one representative experiment.

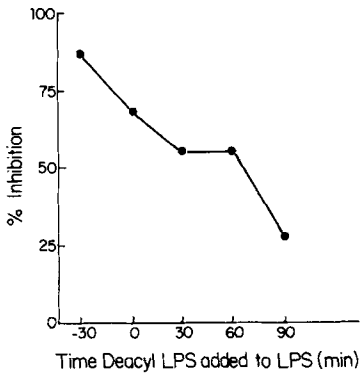


FIGURE 4. Neutrophil adherence to LPS-pretreated HUVE is inhibited when deacylated LPS is added after LPS. HUVE were pretreated with LPS (13.6 ng/ml) for 4 h. Deacylated LPS (268 ng/ml) was added to HUVE with LPS (0), or at various times before (−) or after (+) LPS was added. At the end of the 4-h incubation period, HUVE were washed twice and $^{51}$Cr-labeled neutrophils were added. Percent neutrophil adherence was determined after a 30-min incubation. Percent inhibition represents the reduction of neutrophil adherence to HUVE pretreated with both deacylated LPS and LPS compared with adherence to HUVE pretreated only with LPS.

HUVE. Monophosphoryl lipid A, however, did not reduce neutrophil adherence induced by pretreatment of HUVE with LPS, and lipid X reduced adherence minimally (Table II).

As additional controls, the deacylation reaction mixture was incubated with and without enzyme, treated with ethanol, and stored as described for LPS and deacylated LPS. Pretreatment of HUVE for 4 h with the deacylation reaction mixture or the reaction mixture plus enzyme did not increase subsequent neutrophil adherence, and the reaction mixture or the reaction mixture plus enzyme did not inhibit neutrophil adherence to HUVE induced by LPS (data not shown).

## Discussion

Much is known about the chemical structure of LPS, including the nature of the long-chain fatty acids that are attached by ester and amide linkages to the diglucosamine backbone of the lipid A moiety (22). Typical of LPS from Enterobacteriaceae, *Salmonella typhimurium* LPS has 3-hydroxytetradecanoyl (3-OH-14:0) residues esterified to the 3′ and 3 positions, and amide linked to the 2′ and 2 positions, of the diglucosamine backbone of the lipid A moiety (23). In addition, the hydroxyl groups of the 3-OH-14:0 residues on the nonreducing sugar of the diglucosamine backbone are substituted with nonhydroxylated acyl

TABLE I

*Deacylated LPS Inhibits Neutrophil Adherence to HUVE Pretreated with LPS but not with rhTNF-α or rhIL-1*

| Pretreatment of HUVE | Neutrophil adherence |
|---|---|
| | % |
| Medium | 4 ± 1 |
| Deacylated LPS | 2 ± 1 |
| LPS | 34 ± 1 |
| LPS and deacylated LPS | 14 ± 1* |
| rhTNF-α | 46 ± 9 |
| rhTNF-α and deacylated LPS | 50 ± 8 |
| rhIL-1 | 43 ± 4 |
| rhIL-1 and deacylated LPS | 42 ± 4 |

HUVE were pretreated for 30 min with deacylated LPS (150 ng/ml) or medium. Without washing, medium alone (control) or medium containing LPS (15 ng/ml), rhTNF-α (20 ng/ml), or rhIL-1 (200 pg/ml) was then added. After an additional 4 h incubation, HUVE were then washed and $^{51}$Cr-labeled neutrophils were added. Percent neutrophil adherence was determined after a 30-min incubation. Values represent mean ± SD of four replicate wells.

* $p < 0.001$ compared with LPS.

TABLE II

*Deacylated LPS but not Monophosphoryl Lipid A or Lipid X Inhibits Neutrophil Adherence to HUVE Pretreated with LPS*

| Pretreatment of HUVE | Neutrophil Adherence |
|---|---|
| | % |
| Medium | 2 ± 1 |
| Deacylated LPS | 2 ± 1 |
| Monophosphoryl lipid A | 3 ± 1 |
| Lipid X | 1 ± 1 |
| LPS | 30 ± 4 |
| LPS and deacylated LPS | 3 ± 1* |
| LPS and monophosphoryl lipid A | 28 ± 2 |
| LPS and lipid X | 24 ± 2‡ |

HUVE were pretreated with medium or medium containing deacylated LPS (300 ng/ml), monophosphoryl lipid A (5.0 μg/ml), or lipid X (2.5 μg/ml) for 30 min. Without washing, medium alone or medium containing LPS (15 ng/ml) was then added to HUVE and incubated for an additional 4 h. HUVE were then washed and $^{51}$Cr-labeled neutrophils were added. Percent neutrophil adherence was determined after a 30-min incubation. Values represent means ± SD of four replicate wells.

* $p < 0.001$ compared to LPS.

‡ $p < 0.05$ compared with LPS.

chains. Human neutrophils (10, 13) and macrophages from both normal and endotoxin-hyporesponsive mice (24) have an enzyme (or enzymes) that deacylates *S. typhimurium* LPS by preferential hydrolysis of the acyloxyacyl bonds linking

the nonhydroxylated fatty acids to the 3-OH-14:0 residues. Compared with LPS, deacylated LPS is 100-fold less active in the dermal Shwartzman reaction, although it still retains significant activity as a B cell mitogen (reference 10 and Figure 2). Similar behavior in these tests had been reported previously (25, 26) for lipid A analogs that lack acyloxyacyl groups.

In vitro, LPS induces an endothelial cell surface activity that promotes neutrophil adherence (1, 2). In contrast, pretreatment of HUVE with enzymatically deacylated LPS, lipid X, or monophosphoryl lipid A did not induce neutrophil adherence to HUVE. The inability of these LPS-related compounds to promote neutrophil adherence to endothelium correlates with their failure to provoke the dermal Shwartzman reaction, in agreement with previous evidence (8) that this reaction is, in part, a neutrophil-dependent phenomenon.

Of particular interest was our finding that deacylated LPS completely inhibits LPS-induced neutrophil adherence to HUVE when the ratio of deacylated LPS to LPS (wt/wt) was >20-fold. Deacylated *S. typhimurium* LPS inhibited *E. coli* LPS as well as *S. typhimurium* LPS. The inhibitory effect of deacylated LPS was specific for LPS, since deacylated LPS did not prevent increased neutrophil adherence induced by pretreatment of HUVE with rhIL-1 or rhTNF-$\alpha$.

Complete inhibition of neutrophil adherence to LPS-pretreated endothelium was observed only with deacylated LPS, whereas lipid X and monophosphoryl lipid A (even in large molar excess) did not inhibit adherence. The mechanism by which deacylated LPS inhibits LPS-induced neutrophil adherence to HUVE is unknown. Our data are consistent with the hypothesis that deacylated LPS competitively inhibits the binding of LPS to one or more cell-surface or intracellular targets. Although LPS binding to animal cells has been thought to occur by a nonspecific hydrophobic interaction of the lipid A moiety with cell membranes (27), recent experiments have suggested that macrophages have surface molecules that recognize both the polysaccharide (28) and the lipid A (29) components of LPS. It is thus attractive to speculate that the oligosaccharide chain, which is attached to lipid A in deacylated LPS but not in lipid X or monophosphoryl lipid A, may contribute to the inhibition observed. Studies to characterize the binding of LPS and deacylated LPS to HUVE, as well as to each other, are in progress. Whether deacylated LPS inhibits other inflammatory responses induced by LPS, such as IL-1 or TNF-$\alpha$ production by mononuclear phagocytes, is also currently under investigation.

Duncan and Morrison (30) proposed that processed LPS released from macrophages serves as an immunostimulant to enhance the host response to bacterial invasion. Consistent with this hypothesis is the observation (10) that deacylation of LPS by the leukocyte acyloxyacyl hydrolase dramatically reduces its tissue toxicity while preserving some of its immunostimulatory potential. Our data suggest that deacylation of LPS may further modulate the host response to Gram-negative bacteria by providing a competitive inhibitor of some LPS effects on host target cells.

## Summary

Selective deacylation of the nonhydroxylated fatty acids from *S. typhimurium* LPS by an acyloxyacyl hydrolase isolated from leukocytes reduces toxic activity of LPS in vivo. We examined the effect of deacylated LPS on neutrophil adherence to human umbilical vein endothelial cells (HUVE). Pretreatment of HUVE with LPS (13 ng/ml for 4 h) produced a marked increase in the adherence of subsequently added neutrophils. In contrast, there was no increase in the adherence of neutrophils to HUVE pretreated with deacylated LPS (up to 260 ng/ml for 4 h). Neutrophil adherence to HUVE pretreated with LPS decreased as the degree of LPS deacylation increased.

Deacylated LPS was not only itself inactive, but it inhibited neutrophil-endothelial interactions induced by LPS. Neutrophil adherence to HUVE pretreated with LPS was inhibited by deacylated LPS in a dose-dependent manner. Complete inhibition of adherence was observed at a 20:1 ratio (wt/wt) of deacylated LPS to LPS. Significantly, inhibition of neutrophil adherence to HUVE pretreated with LPS was observed even when deacylated LPS was added to HUVE up to 60 min after LPS. Deacylated LPS, however, did not inhibit neutrophil adherence induced by pretreatment of HUVE with IL-1 or TNF-$\alpha$.

We conclude that enzymatic deacylation of the nonhydroxylated fatty acids of LPS abolishes the ability of LPS to induce surface expression of a neutrophil adherence promoting activity in HUVE. Furthermore, deacylated LPS inhibits neutrophil adherence to HUVE induced by LPS, perhaps by preventing the interaction of LPS with a specific cell-surface or intracellular target.

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