Lipid IV_A Inhibits Synthesis and Release of Tumor Necrosis Factor Induced by Lipopolysaccharide in Human Whole Blood Ex Vivo

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Summary

Tumor necrosis factor (TNF) released by lipopolysaccharide (LPS)-stimulated mononuclear phagocytes is a critical mediator of sepsis. We examined the capacities of rough mutant *Salmonella typhimurium* LPS (Rc) and LPS partial structures lipid A, monophosphoryl lipid A (MPLA), lipid IV_A, and lipid X to induce production of TNF in whole blood. Rc LPS (0.0001–10 ng/ml) produced a dose-dependent release of TNF as determined by cytotoxicity of actinomycin D–sensitized L929 murine fibroblasts. Lipid A, MPLA, lipid IV_A, and lipid X exhibited decreasing capacities to stimulate production of TNF in whole blood, respectively. Fractional deacylation of LPS by incubation with acyloxyacyl hydrolase isolated from human leukocytes produced a reduction in the capacity of LPS to induce TNF release in whole blood. Maximal enzymatic deacylation reduced activity of LPS by >100-fold.

Coincubation with lipid IV_A inhibited TNF release induced by Rc LPS or lipid A, but not by phorbol ester. In contrast, MPLA, lipid X, and deacylated LPS failed to inhibit LPS-stimulated release of TNF. Corresponding to the inhibition of the release of TNF protein, lipid IV_A also inhibited the accumulation of TNF mRNA in LPS-stimulated mononuclear cells. These results suggest that lipid IV_A may act as a competitive antagonist of LPS, perhaps at the receptor level.

Gram-negative bacterial infection induces a complex array of inflammatory responses including fever, leukocytosis, and activation of the coagulation, complement, and kinin systems. These manifestations of Gram-negative infection are primarily induced by heat-stable endotoxins released from the outer membrane of Gram-negative bacteria. LPS are the principal biologically active component of endotoxins that have a common macromolecular architecture. Three major domains comprise LPS structure: an O-antigen side chain composed of repeating oligosaccharides, a core polysaccharide, and lipid A.

Chemical degradation studies and bacterial mutants with defective synthesis of LPS have provided insights into biosynthesis and structure-function relationships of LPS and its components. Lipid A is the smallest substructure of LPS exhibiting all biological activities (1). Lipid A from common pathogens exhibit the same general features, a β-1,6 D-glucosamine disaccharide backbone, with four mole equivalents of 3-hydroxymyristate or 3-hydroxylaurate in amide and ester linkage, and two monophosphate groups at 1 and 4′. Acyl residues are located at the 2, 2′, 3, and 3′ positions on the disaccharides, and the R-3 hydroxyl substitutes of the acyl residues are further esterified with laurate and myristate, forming acyloxyacyl groups. *Escherichia coli* K12 lipid A has 2′ and 3′ acyloxyacyl groups, compared with *Salmonella minnesota*, which may have acyloxyacyl moieties at 2, 2′, and 3′.

The biosynthesis of lipid A involves the formation of acylated monosaccharide precursors that are generated from UDP-*N*-acetylglucosamine (2). Two early intermediates are UDP-2,3-diacylglucosamine and 2,3-diacylglucosamine 1-phosphate (lipid X), which condense to form a tetraacyldisaccharide 1-phosphate with the characteristic β-1,6 linkage (3, 4). The latter metabolite is then phosphorylated at position 4′ to generate lipid IV_A (5) (Fig. 1). Large amounts of lipid IV_A can be isolated from mutants defective in the biosynthesis of 3-deoxy-D-manno-octulosonic acid (6).

Further evaluation of the structurally active components of LPS has come from purification of neutrophil acyloxyacyl hydrolase. This enzyme selectively cleaves the acyloxyacyl bonds found in LPS (7, 8). Hydrolysis of Rc LPS yields a partial-deacylated LPS that displays markedly reduced reactivity in the dermal Shwartzman reaction (9) and fails to stimulate endothelial cells to express proadhesive activity for neutrophils (10), but remains a potent B cell mitogen (9, 10). These

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and other (11–15) studies suggest that a properly acylated diphosphorylated disaccharide is the minimal structure exhibiting full LPS activity.

LPS exerts much of its activity through the generation of cytokines. TNF-α/cachectin is a monokine released in response to LPS stimulation of mononuclear phagocytes. Data from several studies demonstrate that it is a critical mediator of septic shock (16–19). Recently, we have reported a method for evaluating the ex vivo production of TNF in whole blood, the relevant physiologic milieu (20). In this report, we further investigate the ability of LPS and related structures to stimulate production of TNF in whole blood ex vivo. We report that deacylated LPS, monophosphoryl lipid A (MPLA)[1], lipid IV_A, and lipid X all show markedly decreased ability to stimulate TNF production compared with Rc LPS or lipid A. Lipid IV_A and lipid X are significantly less active than MPLA and deacylated LPS. Most importantly, lipid IV_A, but not MPLA or lipid X, inhibits the release of TNF protein and the accumulation of TNF mRNA induced by lipid A or Rc LPS.

Materials and Methods

Reagents. Deacylated LPS preparations were prepared as previously described (7, 8, 21). Briefly, *S. typhimurium* Rc LPS was incubated in a reaction mixture (20 mM Tris citrate, pH 4.8, 150 mM NaCl, 1 mg/ml BSA, 5 mM $CaCl_2$, 0.5% Triton X-100) with and without human neutrophil acyloxyacyl hydrolase. Constant amounts of acyloxyacyl hydrolase and LPS (maintaining a constant ratio) were incubated for varying times, up to 72 h for maximal deacylation (removal of 31–33% of the ^3H-labeled fatty acids from the LPS [9]). The mixtures were then treated with 2 vol of chilled ethanol, precipitating the LPS and BSA, which were then washed once with 80% ethanol. The precipitates were suspended in pyrogen-free saline and stored at −70°C until used.

MPLA (by acid hydrolysis of *S. minnesota* R595) and diphosphoryl lipid A (by hydrolysis at pH 4.5 from *E. coli* K12,D31m4) were obtained from List Biological Laboratories, Inc. (Campbell, CA) and suspended in 0.5% triethylamine-PBS. Lipid X was the gift of Dr. Ingolf Macher, Sandoz Forschungsinstitut (Vienna, Austria), and was suspended in 0.5% triethylamine-PBS with sonication. Lipid IV_A was isolated from *S. typhimurium*, purified as previously described (6), and suspended with sonication in 0.5% triethylamine-PBS. Human recombinant TNF-α (rhTNF) and polyclonal antibody to TNF-α were gifts of Genentech, Inc. (San Francisco, CA). PMA was obtained from Sigma Chemical Co. (St. Louis, MO).

Production of TNF in Whole Blood Ex Vivo. Blood was drawn into polypropylene syringes containing heparin (10 U/ml final concentration). Heparinized whole blood was pipetted into 5-ml polypropylene Falcon tubes (Becton Dickinson & Co., Mountain View, CA) in 225-μl aliquots. Test reagents were diluted in PBS to appropriate concentrations, and 25 μl of the reagent was added per aliquot of whole blood and gently vortexed. The mixture was then incubated at 37°C with constant gentle agitation. After incubation 750 μl of RPMI medium (<0.03 EU/ml; MA Bioproducts, Walkersville, MD) was added, and the cellular component was pelleted by centrifugation at 800 *g* for 15 min. The diluted plasma was removed for assay of TNF.

L929 Cytotoxicity Assay. L929 murine fibroblasts (ATCC CCL1; American Type Culture Collection, Rockville, MD) were grown in 75-cm^2 flasks in RPMI 1640 with 10% horse serum (Flow Laboratories, Inc., McLean, VA). Cells were detached by trypsin, resuspended in growth medium, plated in 96-well flat-bottomed plates (Costar, Cambridge, MA) at 2.5×10^5 cells/well, and grown to confluence at 37°C in 5% CO_2. 50 μl of actinomycin D (4 μg/ml) in RPMI medium containing 5% newborn calf serum (Gibco Laboratories, Grand Island, NY) was added to each well at the time of assay. The test supernatants were added to quadruplicate wells (final volume per well, 200 μl). Control wells were treated with RPMI medium containing 5% newborn calf serum. Maximum cytotoxicity was determined by addition of rhTNF (100 U/ml). After incubation overnight at 37°C, plates were washed three times with PBS with 2% newborn calf serum. Remaining cells were fixed and stained with crystal violet in 20% methanol for 20 min, and then washed with water to remove unbound dye. Stained cells were then lysed with 0.1 M sodium citrate, pH 4.2, in 50% ethanol, and plates were read in a Titertek Multiscan MCC/340 microplate reader (Flow Diagnostics, McLean, VA) at an absorbance of 570 nm (A_{570}). Cytotoxicity was calculated by the following: ratio cytotoxicity = (A_{570} control − A_{570} TNF) − (A_{570} test − A_{570} TNF)/(A_{570} control − A_{570} TNF), where A_{570} control is the absorbance of wells incubated with RPMI/5% sera, A_{570} TNF is absorbance wells incubated 100 U/ml rhTNF, and A_{570} Test is the absorbance of wells incubated with the plasma supernatant from whole blood assay. 1 U of TNF was defined as the amount producing 50% cytotoxicity in the L929 assay.

Northern Blot Analysis. Aliquots of heparinized whole blood were incubated for 4 h at 37°C with sterile saline alone or saline containing Re LPS (10 ng/ml final concentration), lipid IV_A (1,000 ng/ml final concentration), or both Re LPS and lipid IV_A. At the end of the incubation, actinomycin D was then added to final concentration of 20 μg/ml to inhibit further RNA synthesis during subsequent processing. Mononuclear cells were isolated from the whole blood by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation with PBS buffer. The mononuclear cell layer was pelleted at 800 *g* for 15 min. RNA was extracted using a previously described method (21). Briefly, the cell pellet was solubilized in a solution of 5.7 M guanidine hydrochloride and 0.1 M potassium acetate, pH 5.0, and sonicated. A half-volume of 100% ethanol was added and incubated overnight at −30°C. The precipitate was pelleted by centrifugation at 12,000 *g* for 20 min at 4°C, and resuspended in solution of 5.4 M guanidine hydrochloride, 0.1 M potassium acetate, pH 5.0, and 0.025 M EDTA, pH 8.0. DNA was sheared by agitation through a 22-gauge needle and precipitated at −30°C. Final resuspension was in 0.1 M sodium chloride, 0.01 M EDTA, 0.2% SDS, and 0.02 M Tris-hydrochloride, pH 8.0. RNA was extracted twice with equal volumes (0.5 ml) of phenol-chloroform-isoamyl alcohol, then in chloroform-isoamyl alcohol, and precipitated with 100% ethanol. The precipitated RNA (10 μg/lane) was separated by a formaldehyde 1.2% agarose gel and transblotted to nitrocellulose by capillary transfer overnight. The baked blots were prehybridized and then hybridized initially with embryonic chick brain actin cDNA (pA1-pst; 2-kb fragment) to document equal transfer of RNA. They were then stripped and rehybridized with a TNF cDNA probe (a gift of Dr. H. M. Shepard, Genetech, Inc.) and labeled with ^{32}P by multiprime DNA labeling (Amersham Corp., Arlington Heights, IL). Blots were washed and autoradiographs were quantitated by

[1] *Abbreviations used in this paper*: MPLA, monophosphoryl lipid A; rhTNF, recombinant human TNF-α.

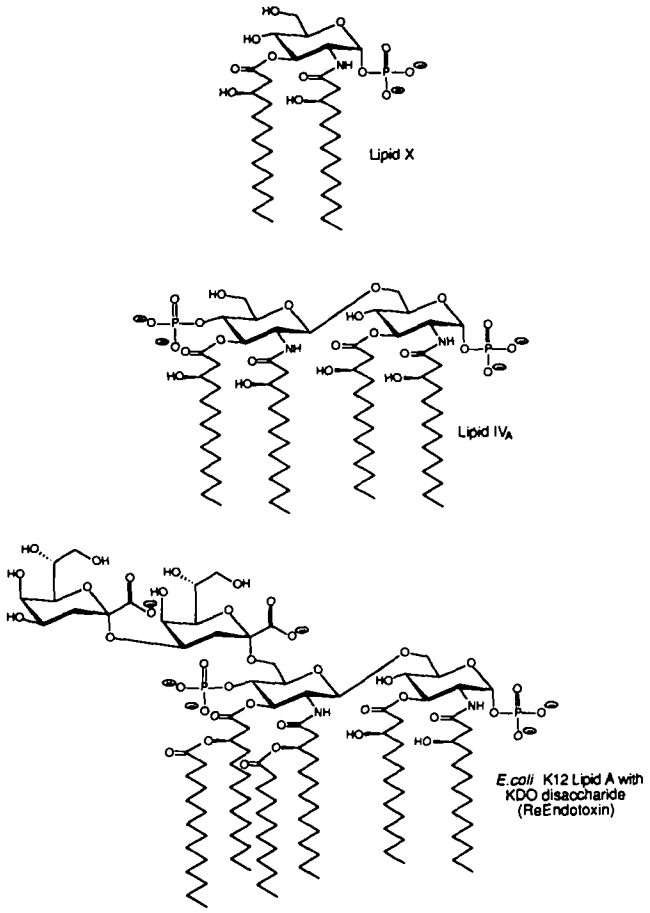

Figure 1. Structures of lipid X, lipid IV_A, and *E. coli* lipid A. Lipid X (2,3-diacylglucosamine 1-phosphate), an early precursor, condenses with UDP-2,3-diacylglucosamine and is phosphorylated at the 4' position forming lipid IV_A. Addition of 6' oligosaccharide core residues, and esterification of laurate or myristate at 2' and 3' positions to form acyloxyacyl groups, yields Rc LPS. *Salmonella* lipid A may be additionally substituted at position 2' to form three acyloxyacyl groups.

laser densitometry (Ultrascan XL; LKB Instruments, Inc., Gaithersburg, MD).

Results

LPS Induces Production of TNF in a Dose-dependent Manner. Addition of Rc LPS (0.0001–10 ng/ml) to whole blood resulted in dose-dependent production of an activity in the diluted plasma that was cytotoxic for actinomycin D–treated L929 murine fibroblasts (Fig. 2). To determine whether the cytotoxic activity was TNF, aliquots of whole blood were stimulated with Rc LPS (0.05 ng/ml) for 6 h, and the diluted plasmas were then incubated for 12 h with RPMI medium, nonspecific rabbit IgG, or polyclonal rabbit antisera to TNF. The polyclonal antibody to TNF abrogated cytotoxic activity (cytotoxicity ratio: RPMI, 0.737 ± 0.042; nonspecific IgG, 0.699 ± 0.041; anti-TNF IgG, 0 ± 0.002; means ± SEM of six experiments).

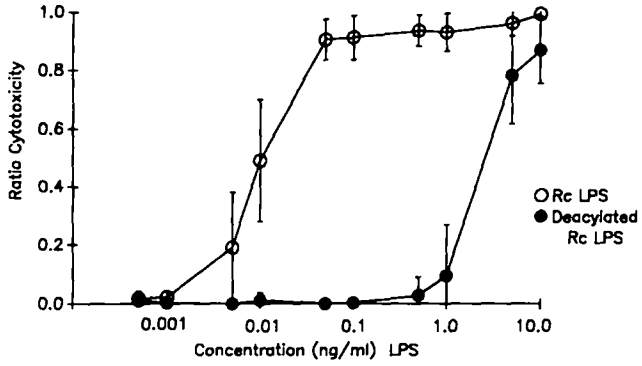

Figure 2. Rc LPS (O) and deacylated Rc LPS (●) induce dose-dependent production of TNF in whole blood. Whole blood was incubated with RPMI medium or RPMI medium containing serial dilutions of Rc LPS or deacylated LPS (0.0001–100 ng/ml final concentration) for 4 h at 37°C. After incubation, the sample was diluted 1:3 with RPMI medium and centrifuged. The supernatant plasma was assayed for TNF. Values represent the means ± SEM of quadruplicate wells in 10 experiments.

Deacylation of LPS Reduces Activity. The ability of deacylated LPS to stimulate production of TNF in whole blood ex vivo TNF was diminished compared with Rc LPS (Fig. 2). Greater than a 100-fold increase (wt/wt) of maximally deacylated LPS relative to Rc LPS was required to induce an equivalent production of TNF. The concentration of LPS required to generate 1 U of TNF (50% cytotoxicity for L929 cells) was 0.022 ± 0.005 ng/ml Rc LPS vs. 3.60 ± 1.18 ng/ml of deacylated LPS (means ± SEM of seven experiments, $p < 0.05$). Fractional deacylation of LPS produced by graded exposure of LPS to the leukocyte acyloxyacyl hydrolase produced a corresponding graded decrease in the capacity of the LPS to induce TNF production (Fig. 3).

Lipid A Precursors and Analogues Exhibit Reduced Activity. Lipid A, MPLA, lipid IV_A, and lipid X exhibited decreasing capacity to stimulate production of TNF in whole blood compared with Rc LPS and Re LPS (Fig. 4 and Table 1). Re LPS was essentially identical to Rc LPS (50% cytotoxicity, 0.037 vs. 0.022 ng/ml). Lipid A was more active than deacylated LPS (50% cytotoxicity, 0.47 vs. 3.60 ng/ml), but demonstrated only one-tenth the activity of Rc LPS. MPLA and lipid IV_A were both markedly less active than Rc LPS and deacylated LPS. Significant cytotoxicity was not observed until the concentration of MPLA was at least 500 ng/ml, and lipid IV_A was 1,000 ng/ml. The capacity of lipid IV_A to induce cytotoxicity at 1,000 ng/ml was variable, with a range of 0.0–0.745 ratio cytotoxicity in 16 separate experiments. In 7 of 16 experiments, a lipid IV_A concentration of 1,000 ng/ml induced no release of TNF. Lipid X was virtually inactive.

Lipid IV_A Inhibits LPS. The dose-dependent production of TNF in whole blood by Rc LPS or lipid A was decreased by coincubation with lipid IV_A (Fig. 5). Complete inhibition was observed at a ratio of lipid IV_A to LPS or lipid A (wt/wt) >100:1 and 50:1, respectively. Deacylated LPS,

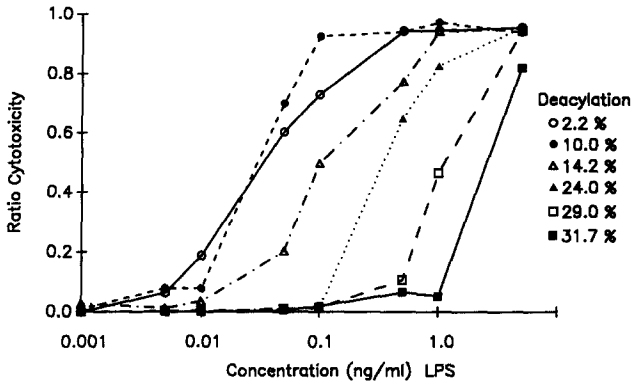

Figure 3. Fractional deacylation of Rc LPS produces a corresponding reduction in the capacity of LPS to stimulate production of TNF. Rc LPS was incubated with acyloxyacyl hydrolase under graded time exposure to produce fractional deacylation of LPS. In this preparation, removal of 31.7% of ^{3}H-labeled fatty acids represented cleavage of >90% of the acyloxyacyl bonds. Dilutions of deacylated LPS preparations in RPMI medium were incubated with whole blood for 4 h at 37°C. After incubation, the samples were diluted 1:3 with RPMI medium and the supernatant plasmas assayed for TNF. Values are the means ± SEM of three separate experiments.

Table 1. *Comparison of LPS Structures*

LPS structure	Concentration producing 50% cytotoxicity
	ng/ml
Rc LPS	0.022
Re LPS	0.037
Lipid A	0.405
Deacylated	3.60
MPLA	454.4
Lipid IV_A	2,656
Lipid X	>5,000

RPMI medium containing serial dilutions of Rc LPS, Re LPS, deacylated LPS, lipid A, lipid IV_A, or lipid X was added to whole blood. After a 6-h incubation at 37°C, samples were diluted 1:3 with RPMI medium. The diluted plasma was collected by centrifugation and assayed for TNF activity by direct cytotoxicity of actinomycin D–sensitized L929 cells. Data from at least four separate experiments were averaged for calculation of cytotoxicity at each dilution. The concentration of LPS structure producing 50% cytotoxicity (1 U TNF) was determined by regression analysis of the linear portion of the averaged dose-response curve.

MPLA, and lipid X failed to inhibit production of TNF by LPS at ratios up to 1,000:1 (Table 2). Maximal inhibition was observed with simultaneous addition of lipid IV_A and LPS. Addition of lipid IV_A 2 h after addition of LPS or lipid A failed to inhibit stimulation of TNF production, with an increasing inhibition as the time interval between additions was shortened. At a ratio of 100:1 (wt/wt), addition of lipid IV_A to whole blood simultaneously with LPS produced 91.6 ± 12.9% inhibition, 15 min after LPS 39.0 ± 20.6% inhibition, 30 min after 15.5 ± 6.4% inhibition, 60 min after LPS 6.0 ± 1.3% inhibition, and 2 h after LPS 1.4 ± 1.3% inhibition (mean percent inhibition ± SEM for three experiments).

PMA (10–100 ng/ml) induced dose-dependent production of TNF in whole blood as determined by L929 cytotoxicity (cytotoxicity ratio at 1 ng/ml, 0.021 ± 0.032; 10 ng/ml, 0.276 ± 0.204; 100 ng/ml, 0.537 ± 0.239; and 1,000 ng/ml,

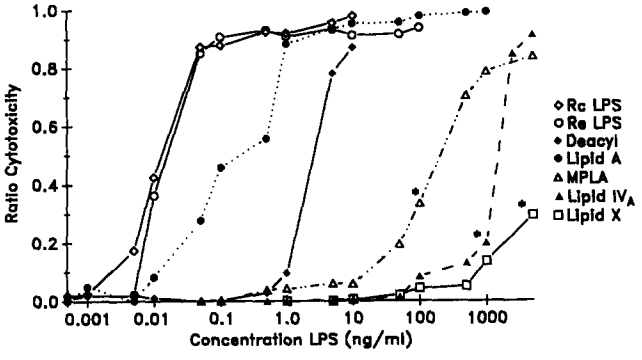

Figure 4. LPS substructures exhibit reduced capacity to induce release of TNF. Serial dilutions of Rc LPS (T) and substructures Re LPS (), deacylated LPS (t), lipid A (●), MPLA (Δ), lipid IV_A (▲), and lipid X (□) were incubated in whole blood for 4 h at 37°C. After incubation, the samples were diluted 1:3 with RPMI medium, centrifuged, and the resultant supernatant plasma was assayed for TNF. Values represent means ± SEM of four or more experiments. Asterisks indicate the lowest concentration of MPLA, lipid IV_A, and lipid X producing significant cytotoxicity ($p < 0.005$ by paired t test).

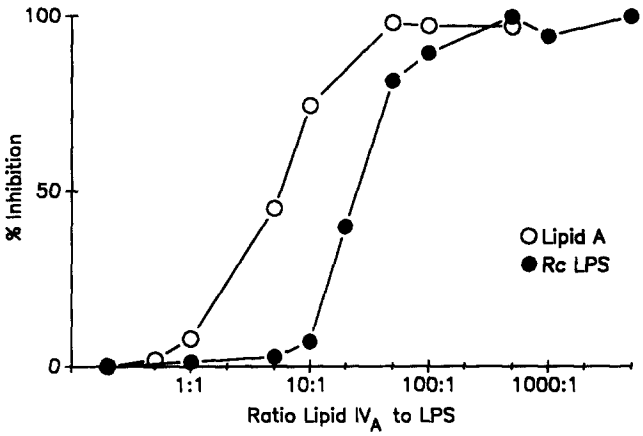

Figure 5. Lipid IV_A inhibits stimulation of TNF production by Rc LPS and lipid A. Whole blood was coincubated with lipid IV_A (1–100 ng/ml) and Rc LPS (●) (0.05–0.1 ng/ml), or lipid A (○) (0.1–1.0 ng/ml), for 4 h. After incubation, the samples were diluted with RPMI medium 1:3, centrifuged, and the supernatant plasmas assayed for TNF. Percent inhibition was calculated as: cytotoxicity (LPS) − cytotoxicity (LPS + lipid IV_A)/cytotoxicity (LPS). Values represent means ± SEM of six experiments.

Table 2. *Effect of Partial Structures on LPS-stimulated Production of TNF*

Coincubation	Percent inhibition
Lipid IV_A	97.2 ± 3.2
Deacylated LPS	0.75 ± 2.8
MPLA	4.8 ± 5.6
Lipid X	0 ± 0

Whole blood was incubated for 6 h at 37°C with Rc LPS (0.01–1 ng/ml) alone or with partial structures and Rc LPS at a ratio 100:1. The concentrations were lipid IV_A, 100 ng/ml; deacylated LPS, 1 ng/ml; MPLA, 10 ng/ml; and lipid X, 100 ng/ml. After incubation, the sample was diluted 1:3 with RPMI medium, centrifuged, and the supernatant diluted plasma assayed for TNF by direct cytotoxicity of actinomycin D–sensitized L929 cells. Percent inhibition was calculated as: 100 × (cytotoxicity Rc LPS − cytotoxicity Rc LPS and precursor)/cytotoxicity with Rc LPS. Values represent the mean ± SEM of four experiments.

0.511 ± 0.219; means ± SEM of three experiments). Equivalent dilutions of PMA in RPMI failed to induce cytotoxicity in the L929 assay (data not shown). Lipid IV_A at ratios up to 10:1 (wt/wt) failed to inhibit stimulation of production of TNF by PMA in whole blood. The cytotoxicity ratio after incubation with PMA alone (10 ng/ml) was 0.276 ± 0.204 vs. 0.445 ± 0.321 when coincubated with lipid IV_A (100 ng/ml; means ± SEM of four experiments). Trials of inhibitory ratios >10:1 were prohibited by lack of significant production of TNF stimulation at PMA concentrations <10 ng/ml, and the slight stimulation by lipid IV_A at concentrations >100 ng/ml.

Lipid IV_A Inhibits Induction of TNF mRNA by LPS. Messenger RNA for TNF was not detected in whole blood incubated with RPMI medium alone. Incubation with Rc LPS (10 ng/ml) induced accumulation of TNF mRNA that was inhibited by coincubation with lipid IV_A (1,000 ng/ml) (Fig. 6). Corresponding production of TNF protein was determined by L929 assay; cytotoxicity ratio with RPMI medium alone was 0.0 ± 0.0; Rc LPS, 0.913 ± 0.017; lipid IV_A, 0.002 ± 0.005; and Rc LPS/lipid IV_A, 0.191 ± 0.057 (average of quadruplicate wells ± SEM for corresponding experiment). Similar results were obtained in three separate experiments comparing accumulation of TNF mRNA and production of TNF protein.

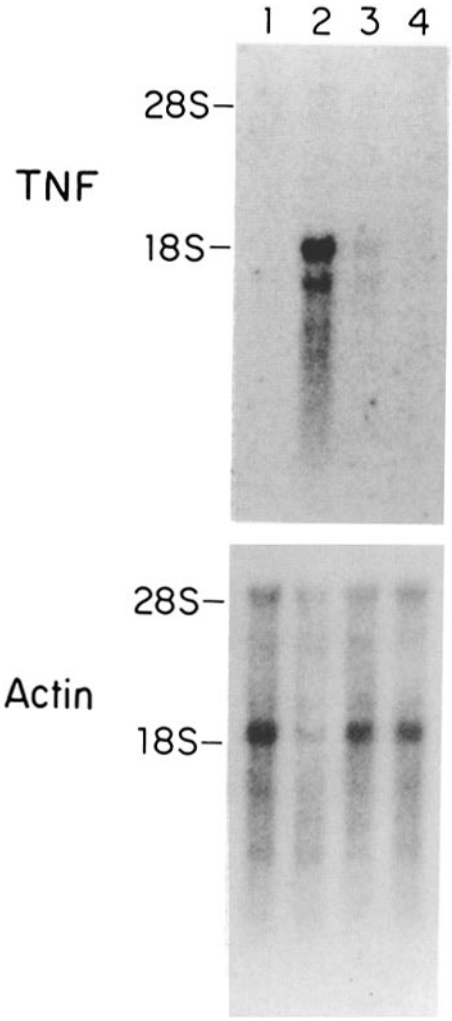

Figure 6. Lipid IV_A inhibits induction of TNF mRNA by LPS. Heparinized whole blood was incubated 4 h at 37°C with sterile saline alone (lane *1*), saline containing Re LPS (10 ng/ml) (lane *2*), lipid IV_A (1,000 ng/ml) (lane *4*), or both Re LPS and lipid IV_A (lane *3*). After incubation, actinomycin D (20 μg/ml) was added to inhibit RNA synthesis during subsequent isolation of the mononuclear cells. RNA was extracted from isolated mononuclear cells as described in Materials and Methods. Total cellular RNA (10 μg/lane) was hybridized to ^{32}P-labeled TNF cDNA or to ^{32}P-labeled chicken β-actin cDNA probe. Ratios of TNF to actin were determined by peak absorbance (AU) of TNF and actin, as measured by laser densitometry.

Discussion

TNF released by LPS-stimulated mononuclear phagocytes is a critical mediator of septic shock (16–19). Recently, we reported a physiologically relevant method of evaluating the production of TNF in whole blood ex vivo (20). In this report, we investigate the structure-function relationships of LPS and its components in the stimulation of TNF release in whole blood.

Rc and Re LPS were potent inducers of TNF release in whole blood. Production of TNF, as determined by cytotoxicity of actinomycin D–sensitized L929 murine fibroblasts, was detectable at concentrations of Rc LPS as low as 5–10 pg/ml. Although lipid A was active in inducing production of TNF, it was not as potent as Rc or Re LPS. Re LPS has been demonstrated to possess enhanced activity in pyrogenicity, Schwartzman reaction, and mitogenicity, but does not exhibit increased lethality over synthetic and natural lipid A preparations (22).

The LPS substructures, MPLA, lipid IV_A, and lipid X,

demonstrated decreasing capacity to induce production of TNF. At the doses of lipid IV_A and lipid X required to induce significant cytotoxicity in the L929 assay (1–10 μg/ml), contamination with trace amounts of LPS remains a possible confounding issue. Less than 0.001% contamination with LPS would be sufficient to provoke release of TNF in the whole blood assay. Both precursors may therefore be even less active than demonstrated. In contrast to their markedly reduced activity in the human whole blood assay, both lipid IV_A and lipid X have been reported to stimulate murine B cell proliferation (23, 24), and to activate the Limulus clotting system (25, 26).

Similar to previous reports of reduced activity in the dermal Shwartzman reaction (9) and in stimulation of endothelial proadhesive activity for neutrophils (10), deacylated LPS was markedly less active than Rc LPS in stimulating TNF release in whole blood. There was a >100-fold decrease (wt/wt) in the ability of deacylated LPS to induce production of TNF compared with properly acylated LPS (Rc). The markedly reduced activity of lipid IV_A compared with lipid A, its properly acylated counterpart, further supports the importance of the acyloxyacyl groups for full induction of TNF production in human cells. In addition, the synthetic analogues of lipid IV_A (compound 406, LA-14-PP) are only weakly pyrogenic, have reduced capacity to elicit leukopenia in rabbits, and are less active in provoking the local Shwartzman reaction compared with a synthetic counterpart (LA-15-PP) of lipid A (11, 27, 28). Induction of IL-1 production by human monocytes by synthetic lipid A is also abrogated by removal of the acyloxyacyl residues (29). However, lipid IV_A is equipotent with lipid A in inducing murine lymphoma κ L chain synthesis (23), and synthetic lipid IV_A analogues are equipotent in activating the Limulus clotting system (26), and provoking lethality in galactosamine-primed mice (11, 27), compared with the synthetic analogue of lipid A. These observations suggest that induction of cytokine production by LPS involves a pathway of activation that requires proper acylation of the LPS backbone, whereas stimulation of other endotoxic activities (activation of murine lymphoma cells or the Limulus clotting system) occurs by pathways that are less sensitive to acylation of lipid A or synthetic analogues.

The significant reduction in the activity of MPLA (prepared by mild hydrolysis of LPS) compared with lipid A also suggests an important role for the 1′ phosphate, the only structural difference between MPLA and lipid A. This supports prior studies in which synthetic analogues of lipid A (LA-17-PP, LA-18-PP) and their monophosphate analogues were active in activating the complement cascade and stimulating the Limulus clotting system, but the monophosphate analogues were inactive in inducing lethality of galactosamine-loaded mice or the production of a serum factor in BCG-primed mice cytocidal to L929 cells (28). The 6′ position is the site of attachment of the underlying disaccharide backbone that forms the core. The possible importance of the core is suggested by the decreased activity of lipid IV_A and lipid A, which lack the KDO groups, compared with their analogous structures, deacylated LPS, and Rc LPS, which retain a core oligosaccharide.

There are, however, alternative explanations for these results. Because the tetraacyldisaccharide backbone is hydrophobic, loss of a phosphate group diminishes the solubility of lipid IV_A and lipid A. Despite suspension in 0.5% triethylamine with sonication, decreased solubility may contribute to the decrease in activity. In addition, residual acyloxyacyl groups not removed by acyloxyacyl hydrolase during production and purification of deacylated LPS could increase the apparent activity of deacylated LPS compared with lipid IV_A. A single acyloxyacyl group confers activity in the dermal Shwartzman reaction (30, 31) and pyrogenicity in rabbits (26).

Of particular interest was our finding that lipid IV_A inhibited production of TNF from human cells by both Rc LPS and lipid A. Lipid IV_A (or precursor Ia [31, 32]) has also recently been shown to inhibit induction of IL-1 by LPS or lipid A in human monocytes (32). In contrast, deacylated LPS, MPLA, and lipid X failed to inhibit production of TNF stimulation by Rc LPS or lipid A. Lipid X, however, has been reported to impart partial protection against a lethal dose of endotoxin administered to mice (33). Deacylated LPS has previously been shown to inhibit the induction of endothelial proadhesive activity for neutrophils by LPS (10), whereas MPLA and lipid X failed to inhibit LPS-stimulated endothelial proadhesiveness under the same conditions. We were unable, however, to produce significant inhibition with deacylated LPS in the whole blood assay. The lack of inhibition by deacylated LPS may again reflect the extreme sensitivity of the whole blood assay to LPS and the possible contamination of the deacylated LPS preparation with residual Rc LPS or incompletely deacylated LPS.

Northern blotting studies demonstrated that the inhibition of LPS-stimulated TNF production by lipid IV_A was associated with diminished accumulation of TNF mRNA. Lipid IV_A could prevent accumulation of the TNF mRNA by pretranscriptional mechanisms (inhibition of signal transduction), by inhibiting transcription, or by promoting degradation of transcripts. The failure of lipid IV_A to inhibit TNF production in response to PMA, however, argues against a nonspecific effect on transcriptional or post-transcriptional mechanisms of regulation.

Although these studies do not delineate the precise mechanism by which lipid IV_A inhibits leukocyte activation by LPS, the results are consistent with lipid IV_A acting as a competitive antagonist of LPS binding to a receptor molecule. An LPS-binding protein of 80 kD has been identified in murine splenocytes (34), although it is not yet known whether this protein is involved in signal transduction. ^{32}P-lipid IV_A has also been used as a probe to detect specific binding on the surface of macrophage tumor cells (35). Alternatively, lipid IV_A could prevent binding of LPS to a plasma protein necessary for interaction with monocytes. Serum is required for endotoxin binding to human monocytes (36), and an LPS-binding protein has been identified in mouse serum (37). Recently, an acute phase serum protein

has also been described in humans, rabbits, mice, and rats that binds a variety of LPS types, including Re LPS, deacylated LPS, lipid A, and lipid IV_A (38, 39).

If lipid IV_A can prevent production of TNF induced by endotoxins of Gram-negative organisms in vivo, it may represent a new approach to the therapy of Gram-negative sepsis.

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