Rhodopseudomonas sphaeroides Lipid A Derivatives Block In Vitro Induction of Tumor Necrosis Factor and Endotoxin Tolerance by Smooth Lipopolysaccharide and Monophosphoryl Lipid A

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***Rhodopseudomonas (Rhodobacter) sphaeroides* diphosphoryl lipid A is a relatively inert species of lipid A but has been shown to antagonize the effects of toxic lipopolysaccharide (LPS) both in vivo and in vitro. The antagonist and its monophosphoryl derivative were examined for the ability to block tumor necrosis factor synthesis and reverse tolerance induction in vitro in macrophage cultures stimulated with bioactive preparations of smooth LPS, rough LPS, diphosphoryl lipid A, and monophosphoryl lipid A. Inhibition of agonist activity and reversal of tolerance by these novel penta-acylated lipid A antagonists provides new insight into macrophage-LPS interactions.**

Lipopolysaccharide (LPS) is an amphipathic molecule located in the outer membrane of gram-negative bacteria and is associated with the many pathophysiologic effects of exposure to endotoxin (reviewed in reference 20 and 32). However, a single exposure to relatively low doses of LPS is associated with the institution of a transient state of hyporesponsiveness to challenge with LPS, which is known as "early endotoxin tolerance" (2, 6, 7, 10, 11, 34). In members of the family *Enterobacteriaceae*, the primary substructure associated with LPS-induced toxicity is the diphosphorylated acylated glucosamine disaccharide, lipid A. Although it is inherently heterogeneous, the composition of the LPS and lipid A of a number of species is sufficiently well defined to make studies of structure versus function possible. Careful analysis of the fatty acid composition and phosphorylation patterns of various lipid A species has shown that the most toxic lipid A is the diphosphoryl hexa-acylated form which occurs naturally in *Escherichia coli* (1, 22, 25, 28, 30). Addition of a seventh acyl group, alteration of the number or length of the lipid A fatty acids, and dephosphorylation of the lipid A result in decreased bioactivity of the structure for eukaryotic cells (21, 24, 30).

Lipid A derived from *Rhodopseudomonas sphaeroides* ATCC 17023 (*Rhodobacter sphaeroides*) has been studied extensively, since it has been shown to be nontoxic (27). Its structure has been completely resolved via nuclear magnetic resonance spectroscopy and soft ionization techniques of mass spectrometry (14, 17, 18, 26). The LPS of *R. sphaeroides* contains an unusual penta-acylated lipid A (RsDPLA [Fig. 1C]), which has 3-hydroxydecanoic acids at R1 and R3 rather than the 3-hydroxytetradecanoic acids present in biologically active *E. coli* lipid A. In addition, it possesses an unsaturated bond in a 14-carbon acyloxyacyl group linked to the 14-carbon R2 and a keto group instead of a hydroxyl group on carbon 3 of the C₁₄ fatty acid at R4. RsDPLA has

been shown to block tumor necrosis factor (TNF) induced by *E. coli* rough Re chemotype LPS (ReLPS) in the murine macrophagelike cell line RAW264.7 (29), as well as TNF induced by ReLPS and smooth LPS in the human monocytic THP-1 cell line (differentiated to become macrophagelike in the presence of phorbol esters; 5). RsDPLA also inhibits ReLPS-induced activation of murine pre-B-cell line 70Z/3 (9), blocks LPS-induced interleukin 1 secretion from peritoneal exudate macrophages (16), and has recently been shown to act as an endotoxin antagonist for mice in vivo (16).

In this study, earlier work showing that RsDPLA was minimally bioactive, yet acted as an antagonist to LPS-induced TNF production, was confirmed and extended. RsDPLA inhibited macrophage-derived TNF induced by active species of smooth and rough LPS, as well as that induced by bioactive lipid A and its less toxic monophosphoryl (MPL) derivative. RsDPLA was also found not to induce tolerance in vitro but blocked the induction of tolerance in macrophage cultures by LPS and its derivatives. The monophosphoryl form of *R. sphaeroides* lipid A (RsMPLA) was also assessed for the capacity to function as an antagonist of LPS and its derivatives. Our findings suggest that binding of RsDPLA is facilitated by the presence of the position 1 phosphoryl group of the reducing terminal glucosamine. The use of RsDPLA and RsMPLA to block LPS-induced TNF and tolerance has provided new clues as to the mechanism by which LPS interacts with macrophages.

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MATERIALS AND METHODS

Reagents. Essentially protein-free (<0.008%), phenol-water-extracted *E. coli* K235 smooth LPS (sLPS) was prepared by the method of McIntire et al. (12). Protein-free *E. coli* D31m4 ReLPS was purified as described elsewhere (Fig. 1A) (15). *Salmonella minnesota* R595 diphosphoryl lipid A

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FIG. 1. Chemical structures of the LPS derivatives used in these experiments. KDO, 2-keto-3-deoxyoctulosonic acid.

(DPLA; Fig. 1B) and MPL (21, 24) were purchased from Ribi Immunochem Research, Inc. (Hamilton, Mont.). Both preparations were assayed by the manufacturer and contained <0.25% protein. Both the DPLA and MPL preparations consist primarily of a mixture of hepta-acyl (Fig. 1B) and hexa-acyl forms, but the MPL lacks the phosphoryl group at position 1 of the reducing terminal glucosamine. Stock solutions of all LPS-related compounds were prepared at 1 mg/ml in normal saline containing 0.2% triethylamine and alternately sonicated vigorously and heated to 65°C to increase solubilization. Stock solutions of all LPS-related compounds were prepared at 1 mg/ml in normal saline containing 0.2% triethylamine and alternately sonicated vigorously and heated to 65°C to increase solubilization. Stock solutions were maintained at -20°C until use.

Derivatives of *R. sphaeroides* RsDPLA (Fig. 1C) and RsMPLA were isolated and purified as follows. LPS was extracted from *R. sphaeroides* ATCC 17023 cells by the phenol-chloroform-petroleum ether method of Galanos et al. (4). RsDPLA was prepared by suspending crude LPS at 3 mg/ml in 0.02 M sodium acetate, adjusting the pH to 2.5 with acetic acid, and incubating it for 70 min at 100°C and recovered as described by Qureshi et al. (19). Crude RsDPLA was purified by passage through a DEAE-cellulose acetate column by using a continuous gradient of 0.03 to 0.08 M ammonium acetate in chloroform-methanol-water (2:3:1, vol/vol/vol), and fractions were analyzed via silica gel thin-layer chromatography as described elsewhere (18). *R. sphaeroides* RsMPLA was prepared from the LPS as previously described (19). The RsMPLA penta-acyl fraction was purified by thin-layer chromatography on silica gel H and run in a solvent system of chloroform-methanol-water-ammonia (50:25:4:2, vol/vol/vol) (17). *R. sphaeroides* derivatives were reconstituted from the lyophilized state with pyrogen-free saline and sonicated to ensure solubilization prior to use.

Macrophage isolation and culture. Five- to 6-week-old LPS-responsive (*Lps*⁺) C3H/OuJ mice (Jackson Laboratory, Bar Harbor, Maine) were used as the source of macrophages for all studies. [The experiments reported herein were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, Department of Health, Education, and Welfare publication no. (NIH) 85-23.] Mice were housed in a laminar-flow hood under 12-h light-dark cycles and provided with standard mouse chow and acidified water ad libitum. Peritoneal exudate macrophages were isolated by peritoneal lavage with ice-cold sterile physiologic saline 4 days after intraperitoneal injection of 3 ml of sterile 3% thioglycolate broth as described elsewhere (8). Cells were washed and suspended in RPMI 1640 supplemented with glutamine, penicillin, streptomycin, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) sodium bicarbonate, and 2% heat-inactivated fetal calf serum and cultured at 2×10^5 cells per well in 96-well tissue culture plates (Costar, Cambridge, Mass.) at 37°C and 6% CO₂. Nonadherent cells were removed by being washed twice with fresh medium 20 to 24 h after plating, and the remaining adherent cells were recultured as indicated. Briefly, cells were pretreated with medium or the *R. sphaeroides* derivatives for 2 h at 37°C in 6% CO₂ prior to LPS stimulation. After the 2-h pretreatment, medium only or the indicated LPS derivatives were added to cultures already containing RsDPLA, and cultures were reincubated for an additional 20 h at 37°C in 6% CO₂. Culture supernatants were then harvested for TNF bioassay.

The assay used to detect induction of tolerance in vitro was essentially identical to that originally described by Virca et al. (31). To assess induction of hyporesponsiveness to LPS (e.g., in vitro tolerance), macrophages were first cultured as for TNF production. Following removal of supernatants from cultures, the cells were washed twice with fresh medium and recultured for 1 h prior to challenge with either medium only or 10 ng of smooth *E. coli* K235 LPS per ml. Cells were reincubated for 20 h, after which supernatants were again harvested for assay of TNF activity. Samples for TNF assay were frozen at -20°C prior to determination of TNF activity.

TNF assay. TNF activity in supernatant samples was determined by a standard cytotoxicity assay with actinomycin D-treated L929 fibroblasts (33). At the end of the assay period, the wells were washed, stained with crystal violet, rinsed with tap water, and air dried. Stain in the wells was eluted with 100 μ l of absolute methanol per well, and absorbance was read in an enzyme-linked immunosorbent assay reader (BioTek Instruments, Inc., Winooski, Vt.) at 595 nm. Units of TNF are presented as the reciprocal of the highest supernatant dilution which resulted in 50% lysis of cells multiplied by the dilution factor (i.e., 20) to give a measure of TNF activity in units per milliliter. The lower and upper limits of sensitivity of the assay are 80 U/ml (i.e., 4 U/50 μ l) and 81,920 U/ml (i.e., 4,096 U/50 μ l), respectively (33).

RESULTS

Induction of TNF by RsDPLA and bioactive LPS derivatives. In contrast to an ReLPS isolated from *E. coli*, RsDPLA has been shown previously to induce relatively low levels of TNF secretion from the RAW264.7 macrophage cell line (28). These findings were confirmed and extended (Fig. 2) by using primary macrophage cultures stimulated with hexa-acylated *E. coli* K235 sLPS, *E. coli* D31m4

FIG. 2. TNF induction by RsDPLA versus LPS and LPS derivatives. Adherent peritoneal exudate macrophages were incubated for 20 h with medium alone or the indicated concentrations of RsDPLA, LPS, or LPS derivatives. Each datum point represents the geometric mean $\pm$ the standard error of the mean of three to eight separate experiments.

ReLPS, *S. minnesota* DPLA, and *S. minnesota* MPL. With regard to TNF induction, sLPS is approximately 10-, 100-, and 1,000-fold more potent on a weight basis than ReLPS, DPLA, and MPL, respectively. Even at concentrations as high as 50 μ g/ml, RsDPLA failed to induce macrophages to secrete TNF in vitro.

Blocking of LPS-induced TNF secretion by RsDPLA. The ability of RsDPLA to block competitively the secretion of TNF from peritoneal exudate macrophages treated with sLPS, ReLPS, DPLA, and MPL was found to be consistent with the relative capacity of each derivative to induce TNF (Fig. 3). RsDPLA blocked TNF secretion induced by 1 ng of sLPS per ml (Fig. 3A), 10 ng of ReLPS per ml (data not shown), 100 ng of DPLA per ml (data not shown), and 1,000 ng of MPL per ml (Fig. 3B) equivalently and in a concentration-dependent fashion. Concentrations of sLPS, ReLPS, DPLA, and MPL greater than these were less efficiently blocked by RsDPLA. Again, RsDPLA by itself failed to induce TNF, even at 50 μ g/ml. The monophosphoryl form of the antagonist RsMPLA also blocked TNF secretion induced by 1 ng of sLPS per ml, 10 ng of ReLPS per ml, 100 ng of DPLA per ml (Fig. 4), and 1,000 ng of MPL per ml, although higher concentrations of RsMPLA than RsDPLA were required to block the three bioactive diphosphoryl species. MPL-induced TNF was blocked equivalently by RsDPLA and RsMPLA (data not shown).

Induction of in vitro tolerance by sLPS, MPL, and RsDPLA. Previous studies demonstrated that pre-exposure of the macrophagelike RAW264.7 cell line to sLPS in vitro results in failure of these cells to respond to subsequent stimulation by 10 ng of sLPS per ml 19 h later to produce TNF (31). In these studies, pretreatment of peritoneal exudate macrophages for 20 h with increasing concentrations of sLPS or MPL, but not RsDPLA, resulted in a dose-dependent decrease in TNF production upon restimulation with 10 ng of sLPS per ml. The in vitro tolerances induced by 100 ng

FIG. 3. Blocking of TNF secretion by RsDPLA. The results show competitive blocking of LPS (A)- or MPL (B)-stimulated TNF production by RsDPLA. The results are representative data derived from 1 of 16 separate experiments. Macrophages were pretreated with the indicated concentrations of RsDPLA for 2 h prior to stimulation for 20 h with the indicated concentrations of sLPS (A) and MPL (B). sLPS concentrations in panel A: 0 (■), 0.1 (■), 1.0 (■), and 10 (▨) ng/ml. MPL concentrations in panel B: 0 (■), 100 (■), 1,000 (■), and 10,000 (▨) ng/ml.

of sLPS per ml and 1,000 ng of MPL per ml were comparable. Unlike sLPS or MPL, concentrations of RsDPLA as high as 50 μ g/ml failed to induce tolerance to challenge with 10 ng of sLPS per ml (Fig. 5).

Blocking of induction of tolerance with RsDPLA. We next assessed the capacity of RsDPLA to block induction of in vitro tolerance by sLPS or MPL. As was observed in Fig. 5, in the absence of RsDPLA (Fig. 6, y axis), 20 h of exposure

FIG. 4. Blocking of TNF secretion by RsDPLA versus RsMPLA. Macrophages were pretreated with the indicated concentrations of RsDPLA or RsMPLA for 2 h prior to stimulation with 100 ng of *S. minnesota* DPLA per ml. Cells were reincubated for 20 h prior to sampling. The results shown are representative data from 1 of 16 separate experiments.

to 100 ng of sLPS per ml or 1,000 ng of MPL per ml resulted in nearly equivalent tolerance to a subsequent sLPS challenge. Figure 6 also shows that RsDPLA was a more effective inhibitor of MPL-induced tolerance than of sLPS-induced tolerance.

DISCUSSION

Quantitative analysis of receptor-ligand binding of LPS to specific cell surface proteins has been fraught with difficulties because of nonspecific binding of LPS to cells at high concentrations. Moreover, multiple receptors on macrophages specific for LPS-lipid A or LPS-lipid A-protein complexes have been described (reviewed in reference 13), although it is not clear to what extent each contributes (individually or collectively) to TNF induction. To circumvent these problems, a structural analog of LPS was used to gain further insight into the mechanisms by which sLPS and its derivatives stimulate TNF and induction of tolerance in primary murine macrophages. Recent studies have shown that the lipid A derived from *R. sphaeroides* is relatively inactive (compared with sLPS or ReLPS) and yet acts as a competitive inhibitor of sLPS- or ReLPS-induced phenomena (5, 16, 27–29). Structural analysis of this molecule has led to the hypothesis that within the penta-acylated RsDPLA, the short length of the fatty acids at R1 and R3 contributes to its lack of bioactivity (18, 23). However, the specificity of RsDPLA as an antagonist of bioactive LPS species and derivatives (5, 9, 16, 29), coupled with its failure to inhibit TNF induced by non-LPS-related compounds (5), suggests that RsDPLA acts by competitively inhibiting the binding of LPS or related structures to a common binding protein(s). However, the possibility that RsDPLA competes with LPS for a soluble lipid A-binding protein, rather than surface receptors per se, has not been explored.

FIG. 5. Induction of in vitro tolerance by RsDPLA, sLPS, and MPL. Adherent peritoneal exudate macrophages were incubated for 20 h with medium alone or the indicated concentrations of sLPS, MPL, or RsDPLA. After the 20 h, cells were washed with fresh medium, rested for 1 h, and restimulated with a 10-ng/ml sLPS challenge. Supernatants were collected after a second 20-h incubation. Each datum point represents the geometric mean $\pm$ the standard error of the mean of three to eight separate experiments.

In this study, we sought first to assess the capacity of RsDPLA to inhibit TNF induction by four agonists: sLPS, ReLPS, DPLA, and MPL. RsDPLA failed to induce TNF at any concentration tested. This is in contrast to previous studies using the RAW264.7 cell line, which produces low but detectable amounts of TNF (approximately 1/10 of those induced by ReLPS), and the bioassay employed in this study (29), and indeed, we also saw TNF induced by RsDPLA at high concentrations in RAW264.7 cells. In contrast, all four agonists induced TNF secretion to the same high level (Fig. 2), although sLPS was 10-fold more potent than ReLPS, 100-fold more potent than DPLA, and 1,000-fold more potent than MPL. This differs from the results obtained with the human THP-1 cell line, with which no marked difference in the capacity of ReLPS versus a variety of sLPS species was observed (5). The relative ability of RsDPLA to block the TNF secretion induced by the four agonists was inversely proportional to their ability to induce TNF secretion. These data are consistent with a model in which the relative activity of each agonist is proportional to its relative capacity to bind to LPS receptors on the cell surface and/or a lipid A-binding protein present in the milieu. Thus, in this model, sLPS would bind most strongly to the macrophage and would be the least displaceable with RsDPLA, and conversely, MPL would bind least strongly and would be most readily excluded from binding to the receptor(s) or carrier protein by RsDPLA.

FIG. 6. RsDPLA reverses the tolerance induced by sLPS and MPL. Adherent peritoneal exudate macrophages were pretreated with medium only or the indicated concentrations of RsDPLA for 2 h and then incubated for 20 h with medium alone, sLPS, or MPL. After the 20 h of incubation, cells were washed with fresh medium, rested for 1 h, and restimulated with a 10-ng/ml sLPS challenge. Supernatants were collected after a second-20 h incubation. The results are representative data derived from 1 of 16 separate experiments.

Significant differences in bioactivity have been attributed to the presence or absence of the position 1 phosphoryl group (21). On the basis of our findings (Fig. 2), we hypothesized that removal of this phosphoryl group from RsDPLA might result in a less effective inhibitor of LPS-induced TNF secretion, possibly owing to destabilization of its interaction with the LPS receptor(s) caused by loss of the large negatively charged moiety. Consistent with the finding that MPL is significantly less potent than DPLA with respect to TNF secretion (Fig. 2), RsMPLA is a less effective inhibitor of TNF induction than is RsDPLA (Fig. 4), but only of induction by those agonists which retain the position 1 phosphoryl group. In contrast, RsMPLA was nearly as effective as RsDPLA as an inhibitor of MPL. These data further support the hypothesis that the interaction of LPS or LPS derivatives with the LPS receptor(s) is strengthened by the presence of the position 1 phosphoryl group.

Macrophages have been shown to mediate the induction of early endotoxin tolerance in vivo (3), and both sLPS and MPL have been demonstrated to induce tolerance in vivo (9); however, significantly more MPL than sLPS is required to induce comparable levels of hyposensitivity to LPS challenge (7, 10). Although this result could be attributed to differential clearance of sLPS versus MPL, in vitro data presented here suggest the possibility that differential interaction of LPS or MPL with macrophages, in part, underlies the in vivo findings. Again, the use of RsDPLA to inhibit induction of tolerance in vitro has provided additional insight into the interaction of LPS and related agonists with macrophages. Pretreatment of cultures with RsDPLA failed to alter macrophage responsiveness to a 10-ng/ml sLPS challenge 20 h later. Consistent with the results shown in Fig.

3A, a challenge dose of 10 ng of sLPS per ml was chosen so that the effect of RsDPLA on tolerance induction could be distinguished from the potential carryover inhibition of the challenge dose. Although unable to block the TNF secretion induced by 10 ng of sLPS per ml initially, RsDPLA was able to block the tolerance induced by 100 ng of sLPS per ml (as well as that induced by 1,000 ng of MPL per ml). As was observed with its blocking of agonist-induced TNF secretion (Fig. 3), RsDPLA was better able to reverse tolerance induced by MPL than that induced by sLPS, again suggesting that MPL interacts less avidly with macrophages than does sLPS.

Another noteworthy finding derived from this study is that for each of the four agonists tested, the concentration necessary to induce TNF was ~10- to 100-fold lower than that required to induce tolerance in vitro. The additional finding that RsDPLA could not effectively block TNF induced by 10 ng of sLPS per ml yet effectively blocked tolerance induced by 100 ng of sLPS per ml (compare Fig. 3 and 6) suggests several possibilities. Tolerance induction may require a higher level of receptor occupancy than induction of TNF secretion. If tolerance is due to a receptor blockade, then the failure of RsDPLA to induce in vitro tolerance must relate to either a faster rate of dissociation of the bound inhibitor from the receptor or the interaction of the agonist (but not RsDPLA) with the receptor, resulting in decreased receptor availability, owing to either physical occlusion or down regulation of receptor availability. The effect of RsDPLA may be to decrease the number of available agonist-binding sites.

An interesting phenomenon is also shown in Fig. 6. Although tolerance induced by 100 ng of sLPS per ml was reversed by RsDPLA concentrations of up to 6.25 μg/ml (i.e., TNF secretion upon challenge was high), higher concentrations of inhibitor resulted in apparent reinstitution of tolerance (as evidenced by decreased TNF secretion upon challenge). The reinstituted suppression of TNF secretion suggests that competition for receptor occupancy alone cannot completely account for the observed high-concentration effects. Perhaps at higher agonist and inhibitor concentrations, mixed micelles form which may increase selectively the presentation of the sLPS to the macrophage and ultimately result in reinstitution of tolerance. Further experiments are required to address this possibility.

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REFERENCES

- Brade, H., L. Brade, U. Schade, U. Zahring, O. Holst, H. Kuhn, A. Rozalski, E. Rohrscheidt, and E. T. Rietschel. 1988. Structure, endotoxicity, immunogenicity, and antigenicity of bacterial lipopolysaccharides (endotoxins, O-antigens), p. 17-45. In J. Levin et al. (ed.), *Bacterial endotoxins: pathophysiological effects, clinical significance, and pharmacological control*. Alan R. Liss, Inc., New York.
- Favorite, G. O., and H. R. Morgan. 1942. Effects produced by the intravenous injection in man of a toxic antigenic material derived from *Eberthella typhosa*: clinical, hematological, chemical, and serological studies. *J. Clin. Invest.* 21:589-599.
- Freudenberg, M. A., and C. Galanos. Induction of tolerance to lipopolysaccharide (LPS)-D-galactosamine lethality by pretreat-

- ment with LPS is mediated by macrophages. *Infect. Immun.* **56**:1352-1357.
4. Galanos, C., O. Lüderitz, and O. Westphal. 1969. A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* **9**:245-249.
 5. Golenbock, D., R. Y. Hampton, N. Qureshi, K. Takayama, and C. R. H. Raetz. 1991. Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes. *J. Biol. Chem.* **266**:19490-19498.
 6. Greisman, S. E., and W. E. Woodward. 1965. Mechanisms of endotoxin tolerance. III. The refractory state during continuous intravenous infusions of endotoxin. *J. Exp. Med.* **121**:911-933.
 7. Henricson, B. E., W. R. Benjamin, and S. N. Vogel. 1990. Differential cytokine induction by doses of lipopolysaccharide and monophosphoryl lipid A that result in equivalent early endotoxin tolerance. *Infect. Immun.* **58**:2429-2437.
 8. Hogan, M. M., and S. N. Vogel. 1988. Production of tumor necrosis factor by rIFN-primed C3H/HeJ (*Lps^d*) macrophages requires the presence of lipid A-associated proteins. *J. Immunol.* **141**:4196-4202.
 9. Kirkland, T. N., N. Qureshi, and K. Takayama. 1991. Diphosphoryl lipid A from lipopolysaccharide (LPS) of *Rhodopseudomonas sphaeroides* inhibits activation of 70Z/3 cells by LPS. *Infect. Immun.* **5**:131-136.
 10. Madonna, G. S., J. Peterson, E. E. Ribí, and S. N. Vogel. 1986. Early-phase endotoxin tolerance: induction by a detoxified lipid A derivative, monophosphoryl lipid A. *Infect. Immun.* **52**:6-11.
 11. Madonna, G. S., and S. N. Vogel. 1985. Early endotoxin tolerance is associated with alterations in bone marrow-derived macrophage precursor pools. *J. Immunol.* **135**:3763-3771.
 12. McIntire, F. C., H. W. Sievert, G. H. Barlow, R. A. Finley, and W. Y. Lee. 1987. Chemical, physical, and biological properties of a lipopolysaccharide from *Escherichia coli* K235. *Biochemistry* **6**:2363-2372.
 13. Morrison, D. C. 1990. Diversity of mammalian macromolecules which bind to bacterial lipopolysaccharides, p. 183-189. In A. Nowotny, J. J. Spitzer, and E. J. Ziegler (ed.), *Cellular and molecular aspects of endotoxic reactions*. Elsevier Science Publishers, B. V., Amsterdam.
 14. Qureshi, N., J. P. Honovich, H. Hara, R. J. Cotter, and K. Takayama. 1988. Location of fatty acids in lipid A obtained from lipopolysaccharide of *Rhodopseudomonas sphaeroides* ATCC 17023. *J. Biol. Chem.* **263**:5502-5504.
 15. Qureshi, N., K. Takayama, D. Heller, and C. Fenselau. 1983. Position of ester groups in the lipid A backbone of lipopolysaccharides obtained from *Salmonella typhimurium*. *J. Biol. Chem.* **258**:12947-12951.
 16. Qureshi, N., K. Takayama, and R. Kurtz. 1991. Diphosphoryl lipid A from the nontoxic lipopolysaccharide of *Rhodopseudomonas sphaeroides* is an endotoxin antagonist in mice. *Infect. Immun.* **59**:441-444.
 17. Qureshi, N., K. Takayama, P. Mascagni, J. Honovich, R. Wong, and R. J. Cotter. 1988. Complete structural determination of lipopolysaccharide obtained from deep rough mutant of *Escherichia coli*. Purification by high performance liquid chromatography and direct analysis by plasma desorption mass spectrometry. *J. Biol. Chem.* **263**:11971-11976.
 18. Qureshi, N., K. Takayama, K. C. Meyers, T. N. Kirkland, C. A. Bush, L. Chen, R. Wang, and R. J. Cotter. 1991. Chemical reduction of 3-oxo and unsaturated groups in fatty acids of diphosphoryl lipid A from the lipopolysaccharide of *Rhodopseudomonas sphaeroides*: comparison of biological properties before and after reduction. *J. Biol. Chem.* **266**:6532-6538.
 19. Qureshi, N., K. Takayama, and E. Ribí. 1982. Purification and structural determination of non-toxic lipid A obtained from the lipopolysaccharide of *Salmonella typhimurium*. *J. Biol. Chem.* **257**:11808-11815.
 20. Raetz, C. R. H., R. J. Ulevitch, S. D. Wright, C. H. Sibley, A. Ding, and C. F. Nathan. 1991. Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. *FASEB J.* **5**:2652-2660.
 21. Ribí, E. E. 1984. Beneficial modification of the endotoxin molecule. *J. Biol. Response Modif.* **3**:1-9.
 22. Rietschel, E. T., H. Brade, L. Brade, K. Brandenburg, U. Schade, U. Seydel, U. Zahring, C. Galanos, O. Lüderitz, O. Westphal, H. Labischinski, S. Kusumoto, and T. Shiba. 1987. Lipid A: the endotoxin center of bacterial lipopolysaccharides: relation of chemical structure to biological activity: detection of bacterial endotoxins with the *Limulus* amoebocyte lysate test, p. 25-53. Alan R. Liss, Inc., New York.
 23. Rietschel, E. T., L. Brade, U. Schade, C. Galanos, M. Freudenberg, O. Lüderitz, S. Kusumoto, and T. Shiba. 1987. Endotoxic properties of synthetic pentaacyl lipid A precursor Ib and a structural isomer. *Eur. J. Biochem.* **169**:27-31.
 24. Rietschel, E. T., H. Gottert, O. Lüderitz, and O. Westphal. 1972. Nature and linkages of the fatty acids present in the lipid A component of *Salmonella* lipopolysaccharides. *Eur. J. Biochem.* **28**:166-173.
 25. Rietschel, E. T., U. Schade, M. Jensen, H. W. Wollenweber, O. Lüderitz, and S. G. Greisman. 1982. Bacterial endotoxins: chemical structure, biological activity and role in septicemia. *Scand. J. Infect. Dis. Suppl.* **31**:8-21.
 26. Salimath, P. V., R. N. Tharanathan, J. Weckesser, and H. Mayer. 1983. Structural studies on the nontoxic lipid A from *Rhodopseudomonas sphaeroides* ATCC 17023. *Eur. J. Biochem.* **136**:195-200.
 27. Strittmatter, R., J. Weckesser, P. V. Salimath, and C. Galanos. 1983. Nontoxic lipopolysaccharide from *Rhodopseudomonas sphaeroides* ATCC 17023. *J. Bacteriol.* **155**:153-158.
 28. Takada, H., and S. Kotani. 1989. Structural requirements of lipid A for endotoxicity and other biological activities. *Crit. Rev. Microbiol.* **16**:477-523.
 29. Takayama, K., N. Qureshi, B. Beutler, and T. N. Kirkland. 1989. The diphosphoryl lipid A from *Rhodopseudomonas sphaeroides* ATCC 17023 blocks induction of cachectin in macrophages by lipopolysaccharide. *Infect. Immun.* **57**:1336-1339.
 30. Takayama, K., N. Qureshi, C. R. H. Raetz, E. E. Ribí, J. Peterson, J. L. Cantrell, F. C. Pearson, J. Wiggins, and A. G. Johnson. 1984. Influence of fine structure of lipid A on *Limulus* amoebocyte lysate clotting and toxic activities. *Infect. Immun.* **45**:350-355.
 31. Virca, G. D., S. Y. Kim, K. B. Glaser, and R. J. Ulevitch. 1989. Lipopolysaccharide induces hyporesponsiveness to its own action in RAW 264.7 cells. *J. Biol. Chem.* **264**:21951-21956.
 32. Vogel, S. N. 1992. The LPS gene: insights into the genetic and molecular basis of LPS responsiveness and macrophage differentiation, p. 485-513. In B. Beutler (ed.), *Tumor necrosis factors: the molecules and their emerging role in medicine*. Raven Press, New York.
 33. Vogel, S. N., and M. M. Hogan. 1991. Measurement of TNF α/β , p. 6.10.1-6.10.5. In J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.), *Current protocols in immunology*. Green Publishing and Wiley-Interscience, New York.
 34. Wharton, D. R. A., and H. J. Creech. 1949. Further studies on the immunological properties of polysaccharide from *Serratia marcescens* (*Bacillus prodigiosus*). II. Nature of the antigenic action and the antibody response in mice. *J. Immunol.* **62**:135-151.