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Diphosphoryl Lipid A Obtained from the Nontoxic Lipopolysaccharide of *Rhodopseudomonas sphaeroides* Is an Endotoxin Antagonist in Mice

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Diphosphoryl lipid A (DPLA) obtained from the nontoxic lipopolysaccharide (LPS) of *Rhodopseudomonas sphaeroides* ATCC 17023 did not induce interleukin-1 release by murine peritoneal macrophages. However, it blocked this induction by toxic deep-rough chemotype LPS (ReLPS) from *Escherichia coli* D31m4. Previously, we obtained similar results on the induction of tumor necrosis factor (TNF) by macrophages. These results showed that DPLA is able to block in vitro the induction of two important mediators of gram-negative bacterial sepsis. We then wanted to determine whether DPLA could also block the induction of TNF by LPS in animals. Mice were treated with 100 μg of *R. sphaeroides* DPLA and challenged 60 min later with 1.0 μg of ReLPS from *E. coli*. The serum TNF level was measured after 60 min. Treatment of mice with this DPLA blocked the rapid and transient rise of TNF caused by ReLPS. This result suggested that *R. sphaeroides* DPLA might be able to protect animals against endotoxin shock caused by gram-negative bacterial infection.

The outer surface of the outer membrane of gram-negative bacteria is occupied by a complex amphipathic molecule called lipopolysaccharide (LPS) (22). LPS triggers many of the pathophysiological events associated with gram-negative bacterial sepsis (10, 13). The events leading to septic shock are thought to be caused primarily by the release of two cytokines, interleukin 1 (IL-1) (4, 11) and tumor necrosis factor (TNF) (2, 11), by LPS-activated mononuclear phagocytes. The lipid A moiety of the LPS is responsible for this activation process (28). The most active form of the free lipid A is the diphosphoryl lipid A (DPLA), with six fatty acyl groups. It is obtained from the LPS of *Escherichia coli* and *Salmonella* strains (28). Its structure is well established (6, 16, 17, 19, 27) (Fig. 1A). Lipid A with either a higher or lower fatty acyl content and a single phosphate group is generally less active (20, 28).

Strittmatter et al. (25) showed that the LPS obtained from *Rhodopseudomonas sphaeroides* ATCC 17023 is 10,000-fold less toxic in galactosamine-sensitized mice and 163-fold less pyrogenic in rabbits than that obtained from *Salmonella abortus-equi*. Takayama et al. (26) obtained DPLA from this LPS (Rs-DPLA) by mild acid hydrolysis and tested it for a key biological activity. The structure of this nontoxic lipid A has been established (15). In contrast to DPLA from *E. coli*, this DPLA could not induce TNF in the RAW 264.7 macrophage cell line, but blocked TNF induction by toxic deep-rough chemotype LPS (ReLPS) from *E. coli* (26). Thus, Rs-DPLA might be unique in that it has no cytokine-producing activity. We have investigated this possibility.

We will show that Rs-DPLA also does not induce IL-1 release by murine macrophages. Additionally, it antagonizes the induction of IL-1 release by ReLPS. We will also show that treatment of mice with Rs-DPLA prevents the rise in TNF level in serum caused by ReLPS.

R. sphaeroides ATCC 17023 was grown photoheterotrophically in medium 550, and LPS was extracted as described previously (15). LPS (640 mg) from *R. sphaeroides* was suspended in 0.02 M sodium acetate, pH 2.5, for 70 min at 100°C. The Rs-DPLA was extracted and purified as described by Qureshi et al. (19a). Briefly, crude Rs-DPLA was fractionated on a DEAE-cellulose column with a linear gradient of 0.03 to 0.08 M ammonium acetate in chloroform-methanol-water (2:3:1, by volume). The desired DPLA eluted off the column about midway through the gradient. The structure of this DPLA was established as shown in Fig. 1B (15, 19a, 23). ReLPS was prepared as described by Qureshi et al. (19).

Peritoneal exudate cells were harvested from BDF_1 mice 48 h after an intraperitoneal injection of thioglycolate. Elicited macrophages were obtained as described previously (9). Macrophages were either pretreated with Rs-DPLA (0.1 to 10 μg/ml) and ReLPS (0.1 μg) was added 2 h later, or they were immediately stimulated with ReLPS (0.01 to 1.00 μg/ml). Controls contained 10 μl of medium containing 0.5% triethylamine. Cultures were incubated at 37°C in 5% CO_2 for 18 h, after which the supernatants were collected and frozen at −20°C until assayed.

IL-1 activity was determined by the comitogenic thymocyte assay (7). C3H/HeJ mice were killed, and the thymus was removed and minced in RPMI 1640 supplemented with 25 μg of gentamicin per ml, 0.225% sodium bicarbonate, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 4 mM glutamine, and 2.5×10^{-5} M β-mercaptoethanol plus 10% heat-inactivated (60°C for 30 min) fetal bovine serum (RPMI-S). Single-cell thymocyte suspensions were then washed twice by centrifugation ($300 \times g$ for 7 min) in RPMI-S. Thymocytes (7×10^5 per well), phytohemagglutinin (0.4 μg), and the samples to be assayed (0.05 ml) were added to flat-bottomed, 96-well tissue culture plates (Costar, Cambridge, Mass.) and incubated for 72 h at 39°C in 5% CO_2. The cells were pulsed with [^{3}H]thymidine for the last 6 h of culture and then harvested onto glass fiber filter paper. [^{3}H]thymidine incorporation was measured by liquid

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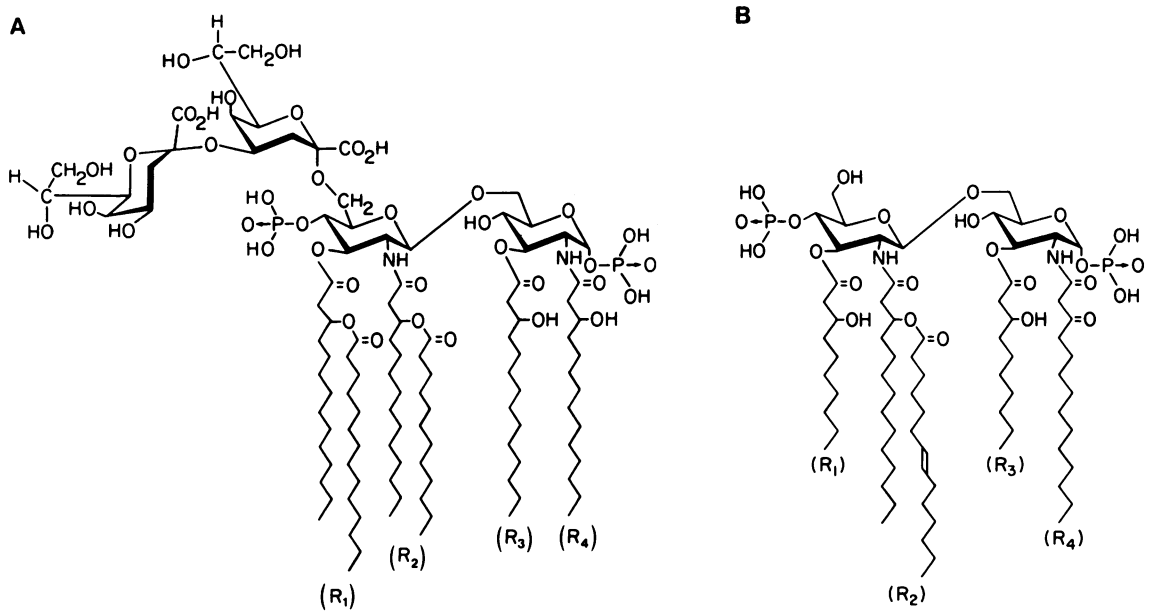


FIG. 1. Structure of (A) ReLPS from *E. coli* D31m4 and (B) one of two predominant structural forms of Rs-DPLA. The other form of Rs-DPLA lacks the double bond in R_2. The *cis*- or *trans*-configuration of the double bond in R_2 is not established. The structure of *E. coli* DPLA lacks the 2-keto-3-deoxyoctonate disaccharide attached to the 6′ position of the glucosamine disaccharide.

scintillation spectrophotometry. Rs-DPLA tested at 0.1, 1.0, and 10 μg/ml was unable to induce IL-1 in murine peritoneal macrophages, in contrast to ReLPS, which gave maximum induction at 0.1 μg/ml (58,565 ± 2,432 cpm).

In the blocking experiment, Rs-DPLA (0.1, 1.0, or 10 μg/ml) was added to the cells 2 h before addition of ReLPS (0.1 μg/ml). Figure 2 shows that Rs-DPLA at 1.0 μg/ml (Rs-DPLA/ReLPS mass ratio of 10:1) inhibited induction of IL-1 release by 60%. When this ratio was increased to 100:1, inhibition was >90%. Rs-DPLA had no endotoxin activity yet showed antagonistic activity in the induction of IL-1 in murine macrophages by ReLPS.

We then wanted to show that the Rs-DPLA is able to block the induction of TNF by ReLPS in animals. For this experiment, we chose the animal model system described by Zuckerman and Bendele (29). Briefly, 12 female BALB/c mice (8 to 12 weeks old) were divided into four groups. The first group received phosphate-buffered saline (PBS) at −60 min and 1.0 μg of ReLPS at 0 min. The second group was pretreated with 100 μg of Rs-DPLA (Rs-DPLA was dissolved in PBS containing 0.5% triethylamine) at −60 min and injected with 1.0 μg of ReLPS at 0 min. The third group received PBS at −60 min and 0 min, and the fourth group received Rs-DPLA at −60 min and PBS at 0 min. All control groups received PBS that contained 0.5% triethylamine. All mice were bled by cardiac puncture at +60 min for quantitation of TNF in serum by the murine L929 fibroblast toxicity assay (5).

ReLPS caused a rise in TNF level to 2,653 ± 286 pg/ml. This increase was blocked by prior treatment of mice with 100 μg of Rs-DPLA (246 ± 95 pg/ml, group 2; 91% inhibition, Fig. 3). Control groups 3 and 4 showed <100 pg of TNF per ml of serum. This experiment was repeated twice and clearly showed that Rs-DPLA blocks the induction of TNF in mice by toxic LPS. When ReLPS and Rs-DPLA were injected simultaneously in a separate but similar experiment, TNF production was inhibited by greater than 80% (5a). Similar results were obtained with guinea pigs. ReLPS (10 μg/450 g) caused a rise in TNF level to 3,000 ± 283 pg/ml. Pretreatment with Rs-DPLA (1.0 mg) at −30 min blocked this rise by 96% (123 ± 59 pg/ml). These results preclude the tolerance phenomenon.

The chemical structure of the ReLPS used as the agonist in induction of IL-1 in murine peritoneal macrophages and of TNF in animal studies is shown in Fig. 1A. The endotoxic moiety is the DPLA structure (28). This structure is compared with the structure of the prominent pentaacyl Rs-DPLA (Fig. 1B), which was an effective antagonist in the biological assays. There are important differences in the two lipid A structures (15, 19, 23). Rs-DPLA has five fatty acids, whereas ReLPS has six; Rs-DPLA has considerably shorter chain-length fatty acids at the 3 and 3′ positions (3-hydroxydecanoic acid at R_1 and R_3) rather than the 3-hydroxytetradecanoic acid in ReLPS; Rs-DPLA has a 3-ketotetradecanoic acid at the 2 position (R_4), whereas ReLPS has a 3-hydroxytetradecanoic acid; and Rs-DPLA has an unsaturated fatty acid in the acyloxyacyl group at the 2′ position (Δ^7-tetradecanoic acid at R_2), versus all saturated fatty acids in ReLPS. A multiple structural requirement might exist for the lack of endotoxicity of Rs-DPLA. A detailed structure-function study is in progress.

Only three valid examples of other LPS antagonists have been reported in the literature (3, 8, 12, 14). Lipid X cannot be considered an LPS antagonist because of the results of a recent study (1). Loppnow et al. showed that the disaccharide lipid A precursor IVA (also called Ia) competes with LPS in the IL-1 assay in human monocytes (8). However, since IVA activates both 70Z/3 pre-B cells (24) and macrophages of murine origin, it could not be used as an antagonist in these systems. Pohlman et al. (12) and Riedo et al. (21) reported that the deacylated LPS prepared by enzymatic hydrolysis of the fatty acyl groups by acyloxyacyl hydrolase (producing LPS that lacks the acyloxyacyl group) inhibits neutrophil adhesion to human endothelial cells and several

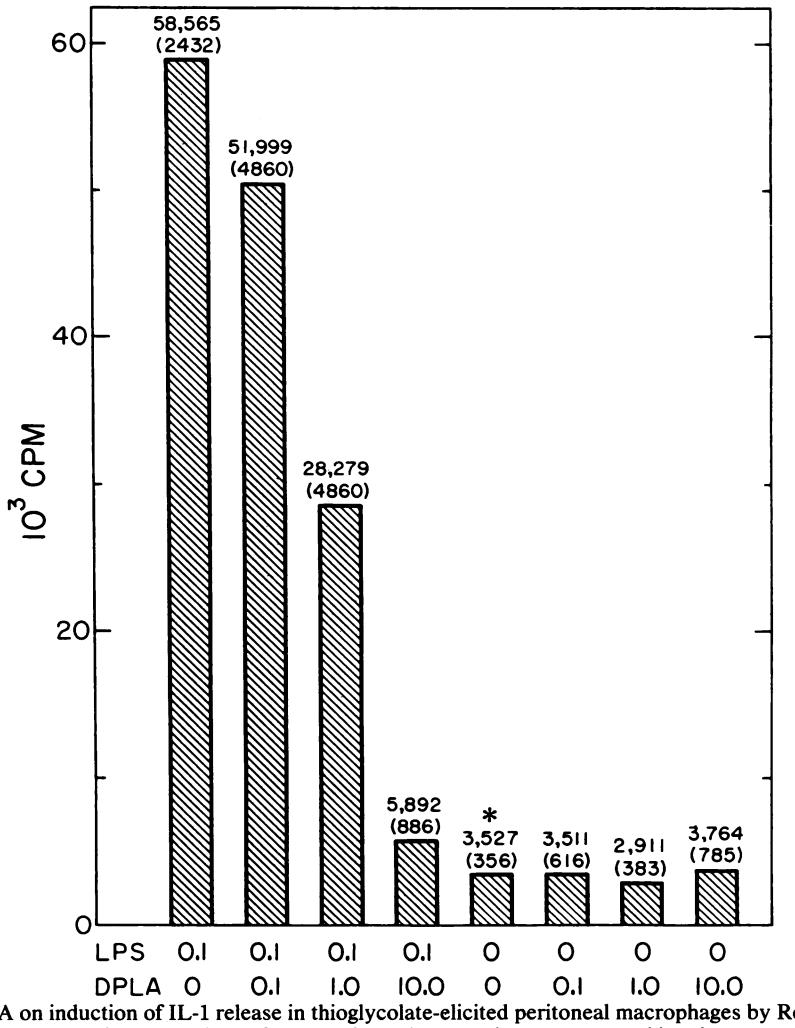
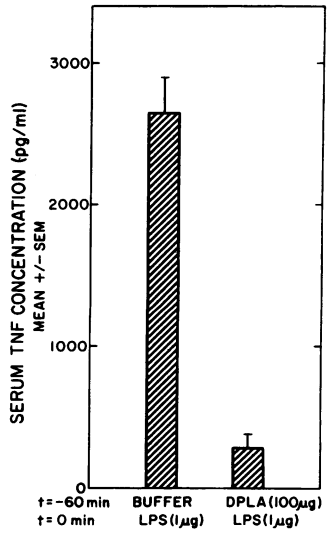


FIG. 2. Effects of Rs-DPLA on induction of IL-1 release in thioglycolate-elicited peritoneal macrophages by ReLPS. ReLPS (0.1 μg) was added to the culture 2 h after Rs-DPLA. Concentrations of the agonist and antagonist are expressed in micrograms per milliliter. Assays were done in triplicate and mean values, standard deviation, and standard error of the mean were calculated. Standard deviations are shown in parentheses. The asterisk (*) represents the control. The control value was slightly higher in the presence of triethylamine.



other responses by the latter cells caused by LPS. It should be noted that the lipid A moiety in these LPS preparations resembles the IVA structure. Thus, the IVA structure in lipid A appears to confer antagonism to LPS in human cells and activation in murine cells.

Rs-DPLA is a uniquely effective antagonist in the LPS activation of B lymphocytes and macrophages of both murine and human origin (18). We suggest that the antagonistic activity of Rs-DPLA could extend to other LPS-responsive cells.

The pentaacyl DPLA obtained from LPS of *R. sphaeroides* is the first lipid A structure reported to show no endotoxic activity and yet to be an effective antagonist of LPS-induced activation of macrophages and B lymphocytes.

FIG. 3. Effects of pretreatment with Rs-DPLA on TNF levels in serum of female BALB/c mice challenged with ReLPS. The Rs-DPLA pretreatment dose was 100 μg injected intraperitoneally at −60 min. The challenge dose of ReLPS injected intraperitoneally was 1.0 μg at 0 min. TNF levels were measured at +60 min.

This Rs-DPLA would be useful for studying the receptor-LPS interaction. It might be effective in protecting animals against endotoxin shock caused by gram-negative bacterial infection. Such studies are in progress.

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