Diphosphoryl Lipid A Derived from Lipopolysaccharide (LPS) of *Rhodopseudomonas sphaeroides* Inhibits Activation of 70Z/3 Cells by LPS

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Diphosphoryl lipid A derived from nontoxic lipopolysaccharide (LPS) of *Rhodopseudomonas sphaeroides* ATCC 17023 did not stimulate the murine pre-B cell line 70Z/3 to synthesize surface immunoglobulin or κ mRNA. However, it effectively blocked *Escherichia coli* LPS-induced activation of 70Z/3 cells in a concentration-dependent manner. This inhibition was specific only to cells activated by LPS, since it did not inhibit activation of 70Z/3 cells by gamma interferon. Maximal inhibitory effect occurred when the antagonist was added within 2 h before adding the LPS. These results strongly suggested that *R. sphaeroides* diphosphoryl lipid A is competing with *E. coli* LPS for physiological lipid A receptors on the 70Z/3 cells.

The lipopolysaccharide (LPS) obtained from *Rhodopseudomonas sphaeroides* ATCC 17023 was reported to be nontoxic by Strittmatter et al. (22). Qureshi et al. (11) compared the lipid A moiety of this LPS to that of toxic *Escherichia coli* LPS and found it to have fine-structural features that might be responsible for its nontoxic properties. Both structural and biological comparisons were made at the level of the most toxic form of lipid A, diphosphoryl lipid A (DPLA) (24). Takayama et al. (23) found *R. sphaeroides* DPLA (RsDPLA) to be inactive in the induction of tumor necrosis factor by RAW 264.7 murine macrophage cell line but capable of blocking the induction by *E. coli* ReLPS. We have continued the biological comparison and now report on the effect of RsDPLA on another LPS-activating system, B lymphocytes.

In this study, we examined the effect of *R. sphaeroides* LPS and DPLA on the murine pre-B cell line 70Z/3. This cell line is a good system in which to study the immunostimulatory effects of LPS because the cells respond to LPS in a reproducible, dose-dependent manner (5, 8). Furthermore, many of the intracellular events after LPS activation in this cell line have been elucidated. LPS causes dissociation of the DNA-binding protein NF-κB from an inhibitor in the cytoplasm, which allows NF-κB to bind to a κB enhancer region within the immunoglobulin light-chain gene (1, 19). Binding of NF-κB to the κB site stimulates synthesis of κ mRNA (7, 19). The cells constitutively produce μ mRNA, and so initiation of κ mRNA synthesis leads to the synthesis of surface immunoglobulin (8, 25). 70Z/3 cells also respond to gamma interferon, although via a different intracellular pathway (2). Because so many aspects of this system are well defined and because this system is a model for some of the immunostimulatory properties of LPS, we decided to investigate the effect of *R. sphaeroides* LPS and lipid A on the activation of 70Z/3 cells. We report that RsDPLA

appears to act as a competitive inhibitor of LPS activation in 70Z/3 cells.

MATERIALS AND METHODS

LPS and lipid A. LPS was prepared from *R. sphaeroides* as previously described (15) and purified on a C18-bonded silica cartridge (Sep-Pak; Waters Associates, Milford, Mass.) (12). The purity of this LPS preparation was assessed by analytical thin-layer chromatography and reverse-phase high-performance liquid chromatography of the methylated derivative (14). DPLA was prepared from purified *R. sphaeroides* LPS by the method of Qureshi et al. (13). Briefly, LPS was hydrolyzed in 0.02 M sodium acetate (pH 2.5) at 100°C for 70 min, and crude DPLA was extracted with chloroform-methanol (2:1 [vol/vol]). This preparation was then fractionated on a DEAE-cellulose column, using an ammonium acetate salt gradient in chloroform-methanol-water (2:3:1 [vol/vol/vol]) to yield highly purified DPLA. Analytical thin-layer chromatography showed this preparation to be at least 99% DPLA, and the distribution of the three major structural forms has already been reported (11). Deep rough LPS (EcReLPS) from *E. coli* mutant D31m4 was purified as previously reported (14).

O-[2-Amino-2-deoxy-N^2-(3-hydroxytetradecanoyl)-β-D-glucopyranosyl]-(1,6)-2-amino-2-deoxy-N^2-(3-hydroxytetradecanoyl)-α-D-glucopyranose 1,4'-bisphosphate (2,2'-DADG) was prepared from purified lipid A precursor IV$_A$ as described below. Precursor IV$_A$ obtained from the temperature-sensitive *kdsA* mutant *Salmonella typhimurium* L50 (21) was suspended in 3.3% (vol/vol) triethylamine at 2 to 3 μg/ml, incubated at 100°C for 60 min, cooled, and lyophilized. This treatment removes the ester-linked fatty acyl groups (15). The sample was dissolved in a small volume of chloroform-methanol (4:1 [vol/vol]) and fractionated in a silicic acid column, using the same solvent. Analytical thin-layer chromatography using silica gel H and two solvent systems of chloroform-methanol-water-concentrated ammo-

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nium hydroxide (50:25:4:2 [vol/vol]) and chloroform–pyridine–88% formic acid–water (40:60:14:5 [vol/vol]) showed that the IV_A was completely converted to a single slower-moving band identified to be 2,2'-DADG. 2,2'-DADG is a diacyl derivative of IV_A which lacks the ester-linked hydroxy-myristate residues at positions 3 and 3'.

Triethylamine salts of LPS or lipid A were prepared by sonicating the LPS or lipid A in 0.1% triethylamine–20 mM EDTA (at 4°C) and dialyzing it overnight against distilled pyrogen-free water. Bovine serum albumin (BSA) complexes were prepared as previously described (5). All experiments were done by using both triethylamine salts of LPS and lipid A and BSA-complexed LPS and lipid A. Identical results were obtained. All the experiments reported here were done with BSA complexes of LPS and lipid A. Gamma interferon was obtained from Amgen (Thousand Oaks, Calif.).

Assay for surface immunoglobulin. 70Z/3 cells were cultured in a total volume of 0.5 ml of RPMI 1640 supplemented with 5% fetal calf serum, 10 mM HEPES (N-2-hydroxyeth-ylpiperazine-N'-2-ethanesulfonic acid), 10 mM glutamine, 5 × 10^{-5} M 2-mercaptoethanol, and 50 μg of gentamicin per ml in a Costar 48-well plate (Costar, Cambridge, Mass.) for 40 h at 37°C. The cells were stained with fluoresceinated-F(ab')$_2$ goat anti-mouse immunoglobulin M (IgM; Tago) and analyzed by flow microfluoremetry as previously described (5). Background fluorescence was determined in 70Z/3 cells cultured without LPS or other inducer and stained with fluoresceinated-F(ab')$_2$ goat anti-mouse IgM. The fluorescence above this background was determined by channel-by-channel subtraction. All assays were done in duplicate, and the standard deviations were less than 10% of the mean. All experiments were repeated at least three times.

Northern (RNA) blot assays. 70Z/3 cells (10^7) were incubated in 5 ml of complete RPMI 1640 in upright Costar 25-cm^2 flasks (3050; Costar). The cells were pelleted by centrifugation, and RNA was harvested with RNAzol B according to the manufacturer's directions (CINNA/BIO-TEX, Friendswood, Tex.). RNA (10 μg per lane) was electrophoresed in an agarose-formaldehyde gel according to published techniques (17). The gel was stained with ethidium bromide to ensure that sharp 18S and 28S RNA bands were seen in each lane. The RNA was transferred to Zeta Bind, and the membrane was baked at 80°C for 2 h. The blots were prehybridized in 50% formamide, 5× Denhardt solution, 1% sodium dodecyl sulfate (SDS), 2% denatured herring sperm DNA, 5× SSPE (17), and 10% dextran sulfate for 8 h at 45°C. The labeled probe was added and allowed to hybridize overnight at 45°C. The blot was then washed at 25°C in 1× SSPE–0.1% SDS for 5 min and at 60°C in 0.1× SSPE–0.1% SDS for a total of 60 min. The filter was air dried and autoradiographed.

LPS stimulation of 70Z/3 cells induces κ-chain mRNA transcription but has little effect on μ-chain transcription (25). Therefore, all blots were hybridized initially with a κ probe and then boiled and hybridized with a probe for the μ chain to ensure that equal amounts of RNA were loaded in each lane. The autoradiographs of both hybridizations are shown. The κ-chain probe was a cDNA constant region probe, pGEM2Cκ (18), which was a gift from Ursula Storb. The 500-bp insert was excised with PstI and purified by agarose gel electrophoresis. The μ probe (used intact) was pμ3741 (16) which was a gift from Roy Riblet. The probes were labeled with the Random Primed DNA Labelling Kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions. The average specific activity
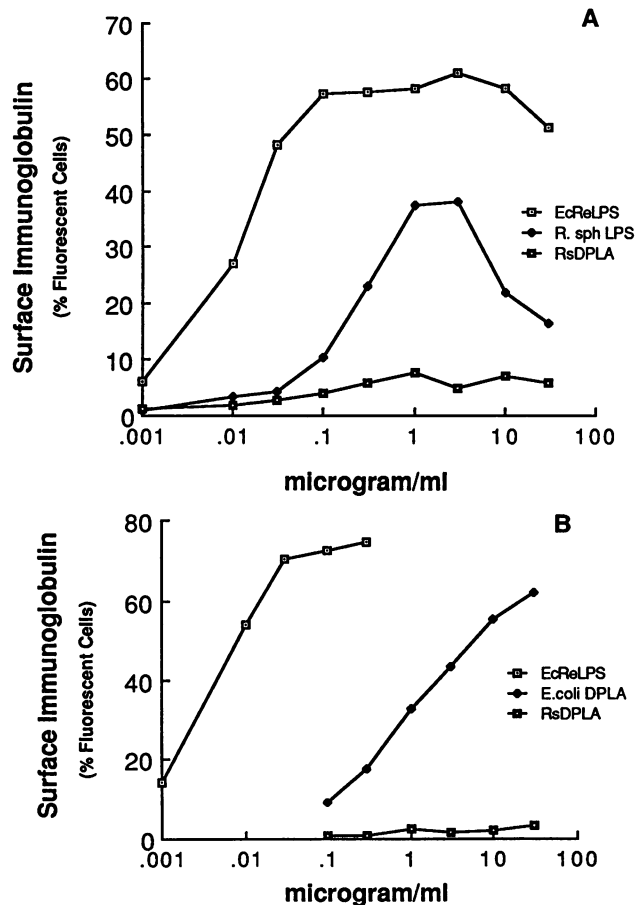

FIG. 1. (A) Activation of 70Z/3 cells by EcReLPS, *R. sphaeroides* LPS, and RsDPLA. The percentage of fluorescent cells is plotted against the concentration of LPS or DPLA. Standard deviations were less than 10% of the mean. (B) Comparison of EcReLPS, *E. coli* DPLA, and RsDPLA.

was approximately 10^9 cpm/μg of DNA. Densitometry was done by using the LKB Ultrascan densitometer.

RESULTS

Figure 1A shows the dose-response curve for *R. sphaeroides* LPS, RsDPLA, and EcReLPS plotted against the percentage of surface immunoglobulin-expressing 70Z/3 cells (percent fluorescent cells). The *R. sphaeroides* LPS was reproducibly active only at the higher concentrations of 0.3 to 1.0 μg/ml. This level of activity was almost 100-fold less than that for EcReLPS. The RsDLPA was essentially inactive over the entire dose range tested. Figure 1B shows the activity of DPLA derived from EcReLPS and *R. sphaeroides* LPS. *E. coli* DPLA was approximately 100-fold less active than EcReLPS; RsDPLA was totally inactive.

Figure 2 shows the effects of *R. sphaeroides* LPS and DPLA on activation of 70Z/3 cells by EcReLPS. 70Z/3 cells were preincubated for 2 h with the indicated concentrations of the *R. sphaeroides* compounds and then exposed to 0.1 μg of EcReLPS per ml for 40 h. At concentrations of 0.1 μg/ml or below, neither *R. sphaeroides* LPS nor DPLA had significant inhibitory effect. At doses of 1, 10, and 30 μg/ml, RsDPLA strongly inhibited the 70Z/3 cell response. *R.*
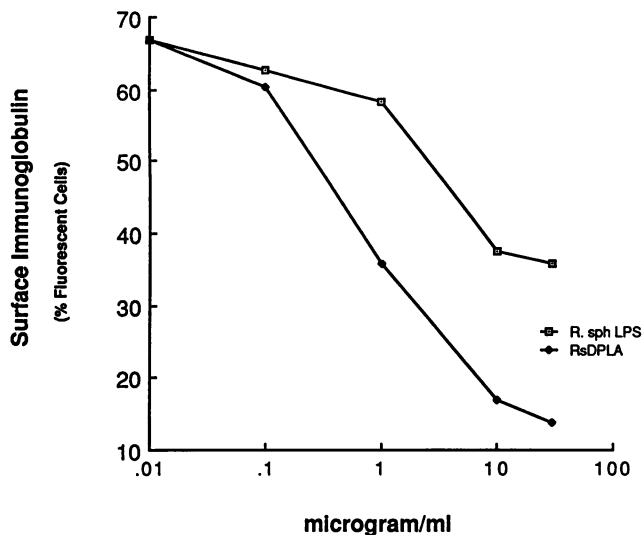
FIG. 2. Effect of *R. sphaeroides* LPS and DPLA on the activation of 70Z/3 cells by 0.1 µg of EcReLPS per ml. The maximum response of 70Z/3 cells was 68%.

sphaeroides LPS was not as effective at inhibiting the response although the intrinsic activity of the *R. sphaeroides* LPS at high concentrations (20% fluorescent cells at 10 µg/ml) may have obscured inhibition by this compound.

We have previously reported that 2,2'-DADG does not stimulate 70Z/3 cells (5). Figure 3A shows typical data demonstrating the lack of activity of 2,2'-DADG and Rs-DPLA. We compared the relative ability of 2,2'-DADG and RsDPLA to inhibit the 70Z/3 cell response to EcReLPS (Fig. 3B). Although both compounds were inhibitory, RsDPLA was 80-fold more effective.

We then asked whether the inhibitory effect of RsDPLA was specific to cells activated by LPS. 70Z/3 cells are known to respond to gamma interferon (2). The effect of RsDPLA on the response of 70Z/3 cells to gamma interferon is shown in Table 1. Concentrations of RsDPLA which completely inhibit the 70Z/3 cell response to EcReLPS had no effect on the response to gamma interferon, suggesting that the inhibitory effect is specific for LPS.

Table 2 shows the relationship between the concentration of EcReLPS used to stimulate a response and the concentration of RsDPLA needed to inhibit the response. In this experiment, 70Z/3 cells were preincubated with the indicated concentrations of RsDPLA and then stimulated with a range of concentrations of EcReLPS. The amount of RsDPLA needed to inhibit the response appeared to depend on the dose of EcReLPS used to stimulate the response (Table 2). For example, 1 µg of RsDPLA per ml inhibited the response to 0.1 µg of EcReLPS per ml by 61%; in contrast, it required
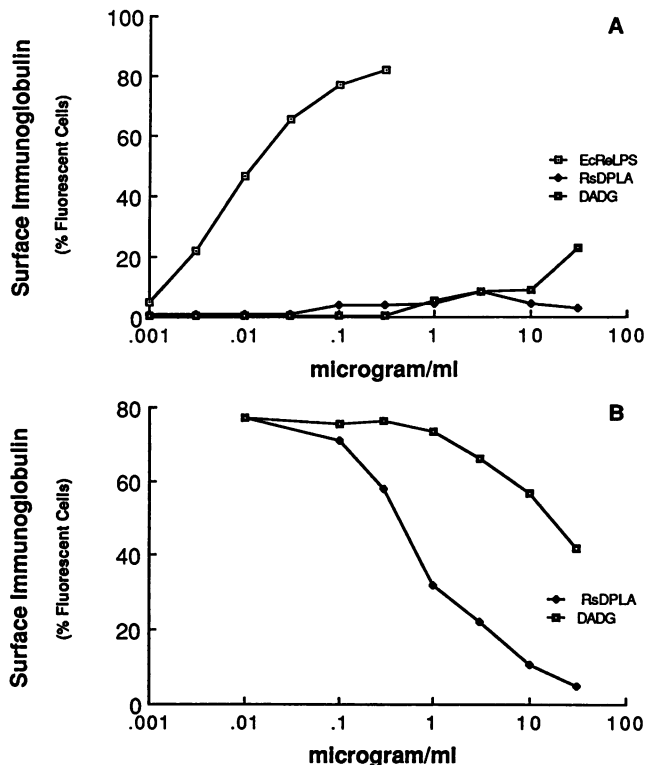

FIG. 3. (A) Activation of 70Z/3 cells by EcReLPS, RsDPLA, and 2,2'-DADG. (B) Effect of *R. sphaeroides* LPS and 2,2'-DADG on the activation of 70Z/3 cells by 0.1 µg of EcReLPS per ml. The maximum response of 70Z/3 cells was 77%.

10 µg of RsDPLA per ml to inhibit the response to 1 µg of EcReLPS per ml to the same degree.

Although RsDPLA did not cause 70Z/3 cells to express surface immunoglobulin, it was possible that RsDPLA did induce κ mRNA synthesis, but not translation of mRNA to protein. 70Z/3 cells were stimulated with 1 µg of EcReLPS or RsDPLA per ml, and the amount of κ-chain mRNA was determined as a function of time. Figure 4 shows that κ-chain mRNA is detectable within 4 h after stimulation with EcReLPS, and the amount steadily increases up to 24 h. RsDPLA does not induce κ mRNA over that period of time. As expected, neither RsDPLA nor EcReLPS had any effect on the steady-state levels of µ mRNA.

The κ mRNA assay was also used to examine the kinetics of inhibition by RsDPLA. A 100-fold excess of RsDPLA added 2 h before EcReLPS completely inhibited the response (Fig. 5). The addition of RsDPLA as late as 8 h after EcReLPS inhibited the response by approximately 50%. If RsDPLA was added 22 h after EcReLPS (2 h before harvesting), it had no effect.

TABLE 1. Comparison of the inhibitory effect of RsDPLA on activation of 70Z/3 cells by EcReLPS and gamma interferon

Stimulus	% Fluorescent cells (% inhibition) with the indicated concn (µg/ml) of RsDPLA[a]					
	0	0.1	0.3	1	3	10
None	0	10.5	13.3	10.1	10	8.5
EcReLPS (0.1 µg/ml)	84.1	75.1 (11)	57.7 (31)	33.2 (61)	25.4 (70)	13.1 (84)
Gamma interferon (1,000 U)	41.6	55 (0)	55.4 (0)	54.1 (0)	52.2 (0)	52.7 (0)

[a] % Fluorescent cells is a measure of activation of 70Z/3 cells. RsDPLA was added 2 h before EcReLPS or gamma interferon. % Inhibition is calculated as percentage of uninhibited sample.

TABLE 2. Concentration-dependent inhibition by RsDPLA of the activation of 70Z/3 cells by EcReLPS

Stimulatory concn (μg/ml) of EcReLPS	% Fluorescent cells (% inhibition) with RsDPLA concn (μg/ml) of[a]:			
	0	1.0	3.0	10
0	0	3.5	2.5	6
0.1	73	28.5 (61)	13.5 (92)	4.5 (94)
0.3	74	48.5 (34)	30 (59)	11 (85)
1	75	57.5 (22)	55 (26)	25 (66)

[a] RsDPLA was added 2 h before EcReLPS. % Inhibition is calculated as percentage of uninhibited sample.

DISCUSSION

In these studies, we have found that RsDPLA does not stimulate 70Z/3 cells and appears to compete with active LPS to prevent cellular activation. The lack of activity of RsDPLA is in agreement with previous reports that LPS and
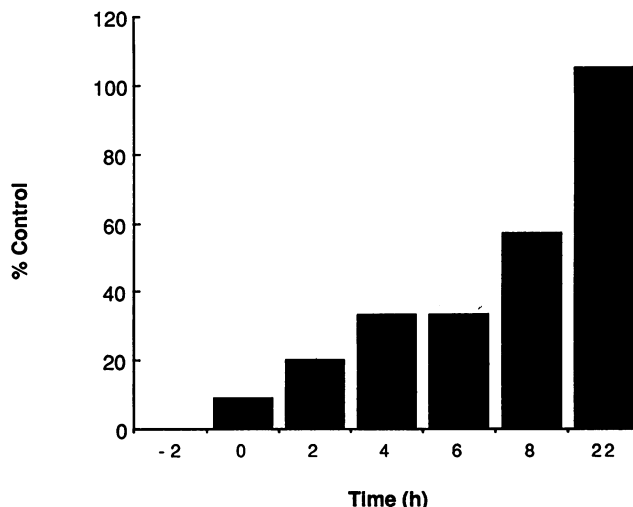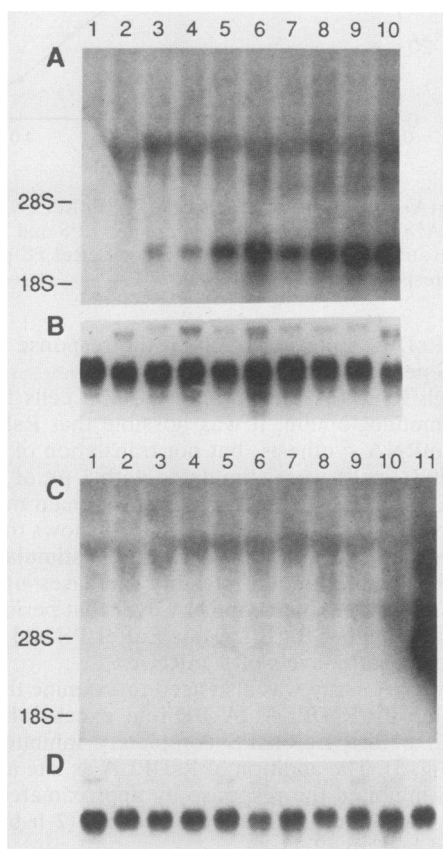

FIG. 4. Induction of κ mRNA synthesis by EcReLPS, but not by RsDPLA. (A and B) 70Z/3 cells were stimulated with 1 μg of EcReLPS per ml for the indicated periods of time (hours): none (lane 1), 2 (lane 2), 4 (lane 3), 6 (lane 4), 8 (lane 5), 10 (lane 6), 12 (lane 7), 14 (lane 8), 18 (lane 9), and 24 (lane 10). The cells were hybridized with the κ probe (whole blot shown) (A) or with the μ probe (hybridizing bands shown) (B). (C and D) 70Z/3 cells were stimulated with 1 μg of RsDPLA per ml (lanes 1 to 10) or 1 μg of D31m4 LPS per ml (lane 11) for the indicated periods of time (hours): none (lane 1), 2 (lane 2), 4 (lane 3), 6 (lane 4), 8 (lane 5), 10 (lane 6), 12 (lane 7), 14 (lane 8), 18 (lane 9), and 24 (lanes 10 and 11). The cells were hybridized with the κ probe (whole blot shown) (C) or with the μ probe (hybridizing bands shown) (D).

FIG. 5. Kinetics of inhibition by RsDPLA. Densitometry of Northern blots presented as a function of time of addition of RsDPLA.

lipid A from *R. sphaeroides* are relatively inactive in several assays of LPS activity (22). This is a remarkable finding, considering the structural similarity of *E. coli* and *R. sphaeroides* lipid A. RsDPLA has a β-D-glucosaminyl-1,6-D-glucosamine-1,4'-bisphosphate disaccharide backbone which is identical to the disaccharide backbone of *E. coli* lipid A. The small differences between *E. coli* and *R. sphaeroides* lipid A are limited to the fatty acids. RsDPLA has hydroxy decanoic acid at positions 3 and 3', a keto tetradecanoic acid at position 2, and a Δ^7-tetradecanoyloxy-tetradecanoic acid at position 2' (Fig. 6) (11). In contrast, *E. coli* lipid A has hydroxytetradecanoic acid at positions 2 and 3 and dodecanoyloxytetradecanoic and tetradecanoyloxytetradecanoic acids at positions 2' and 3', respectively. These structural differences appear sufficient to render the RsDPLA inactive. The differences noted are one less fatty acid and shorter-chain fatty acids in RsDPLA compared with *E. coli* DPLA. The number of fatty acyl groups in lipid A has been shown to be an important factor in determining its biological activities (6). However, we do not think that the difference in the number of fatty acids (five in RsDPLA and six in *E. coli* DPLA) accounts for the difference in activity, because IV_A, which has only four fatty acids, stimulates 70Z/3 cells as well as *E. coli* lipid A (5, 20). We presume that the differences in the chain length of the primary fatty acids (C_{10} and C_{14} in RsDPLA as opposed to C_{14} in *E. coli* DPLA) or other structural features, such as the additional keto group on fatty acid at position 2 in RsDPLA, may account for the biological inactivity of this lipid A.

The RsDPLA (containing five fatty acids) was clearly more effective than 2,2'-DADG (containing only two fatty acids) in blocking the activation of 70Z/3 cells by EcReLPS. Thus, there must be a requirement for the number of fatty acids needed to achieve maximum inhibition. This requirement appears to be greater than two but less than six.

We think that the inhibition of LPS activation of 70Z/3 cells by *R. sphaeroides* lipid A is due to competitive binding to the physiological lipid A receptors. The evidence for this idea is as follows. (i) The ratio of RsDPLA to toxic EcReLPS must be 10:1 to 100:1 to inhibit LPS activation. (ii) RsDPLA has no effect on activation of 70Z/3 cells by gamma interferon. (iii) RsDPLA must be present within the first 2 h of
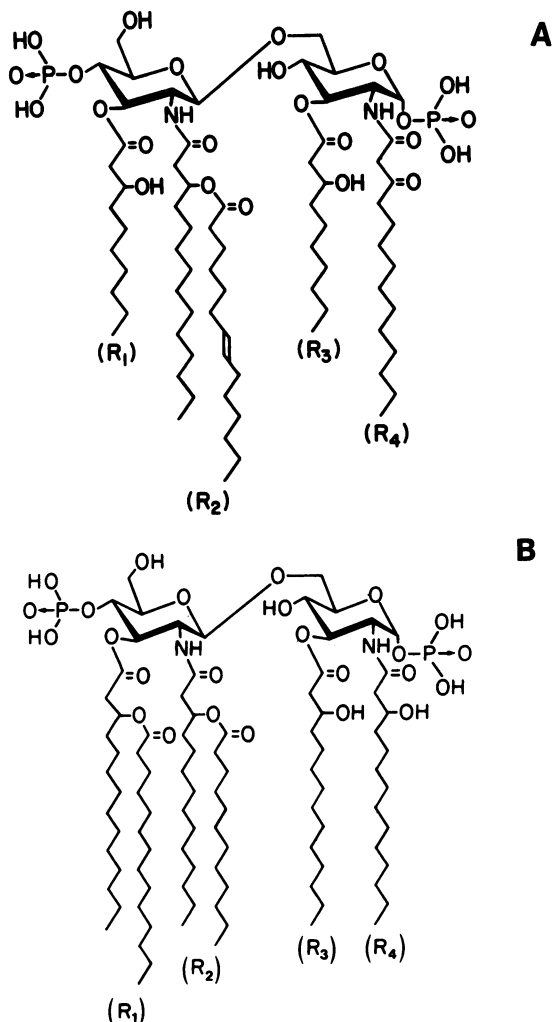
FIG. 6. Structures of RsDPLA (A) and *E. coli* DPLA (B).

the nonhydroxyl fatty acids from LPS renders the product less toxic and effective in inhibiting neutrophil adhesion to endothelial cells caused by LPS. Loppnow et al. (6) have reported that a lipid A precursor lacking secondary fatty acids (termed both IV_A and Ia in the literature) did not stimulate human mononuclear cells to synthesize interleukin-1. This compound was also able to inhibit the LPS-induced interleukin-1 response. Since lipid A precursor IV_A (Ia) stimulates 70Z/3 cells quite well (5, 20) the lack of interleukin-1 response by human monocytes suggests that these cells have different structural requirements for an active lipid A molecule. Whether these differences reflect differences in species, cell type, or degree of differentiation remains to be investigated.

Our data further demonstrate the ability of RsDPLA to inhibit LPS-induced responses in vitro. Whether RsDPLA can function as a competitive inhibitor of LPS toxicity in vivo is another interesting question which remains to be answered.

ACKNOWLEDGMENTS

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LPS stimulation to maximally inhibit the response. Although these results are consistent with the hypothesis that RsDPLA functions as a competitive inhibitor, they do not conclusively prove that point. Cellular binding studies of LPS have not demonstrated saturable binding (26), which makes competitive binding studies of LPS binding difficult to interpret. In other experiments, we have found that proteins (with molecular masses of 18, 25, and 31 kDa by SDS-polyacrylamide gel electrophoresis) are labeled by an ^{125}I-labelled photoaffinity derivative of *Salmonella minnesota* R595 ReLPS (4). Incubation of the cells with an excess of unlabeled LPS or RsDPLA inhibited labeling of these proteins but did not affect the total amount of LPS bound to the cell. For these reasons, we think that LPS binding to the 18-, 25-, and 31-kDa proteins may be of functional importance.

Our results suggesting that RsDPLA is a competitive inhibitor of LPS are consistent with a number of studies in the literature. It has been suggested that lipid X competitively inhibits LPS activation of 70Z/3 cells in vitro (20) and can prevent LPS toxicity in vivo (10). Danner et al. (3) found that lipid X and its analog 3-aza-lipid X inhibited LPS-induced neutrophil priming in vitro. This inhibition appeared to result from competition with LPS for cellular binding sites. Pohlman et al. (9) showed that selective deacylation of

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