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*

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Unlike the diphosphoryl lipid A (DPLA) derived from toxic lipopolysaccharide of Escherichia coli and Salmonella strains, the DPLA from nontoxic lipopolysaccharide of Rhodopseudomonas sphaeroides ATCC 17023 is biologically inactive. This could be due to the presence of 3-oxotetradecanoic and Δ2-tetradecenoic acids. These two fatty acids in R. sphaeroides DPLA were catalytically reduced in platinum oxide/H2 to the 3-hydroxy and saturated fatty acids, respectively. The biologically active E. coli DPLA was also treated with platinum oxide/H2, but as expected, the reduction step did not change the structure. These two preparations were then compared with the untreated samples for biological activity in three select in vitro assays.

Over a range of 0.01–100 ng/ml, both normal and reduced DPLA from R. sphaeroides were inactive in priming phorbol myristate acetate-stimulated superoxide anion release in human alveolar macrophages. Over a range of 10–100 ng/ml, both samples failed to induce tumor necrosis factor in the RAW 264.7 murine macrophage cell line. The reduced DPLA marginally activated 70Z/3 pre-B cells at concentrations of 0.1–30 μg/ml. In every case, both normal and platinum oxide/H2-treated E. coli DPLA were biologically active.

These results indicate that the lack of biological activity of R. sphaeroides DPLA is not due to the presence of 3-oxo and unsaturated fatty acids, but rather to one or more of the following: (i) presence of only five fatty acyl groups (compared to six in active lipid A); (ii) presence of 3-hydroxydecanoic acids (rather than 3-hydroxytetradecanoic, in active lipid A); (iii) greater variation in size of the fatty acids.

The lipopolysaccharides (LPS)1 of Escherichia coli and the Salmonella strains are very endotoxic (1). This property is associated with the lipid A moiety (2–4). It is now possible to isolate and purify the endotoxic moiety in the form of DPLA from the toxic LPS (5, 6). Thus, the model toxic lipid A would be the hexaacyl DPLA obtained from LPS of E. coli and Salmonella strains. This structure has been established (6–10) (Fig. 1 B).

The LPS of Rhodopseudomonas sphaeroides has been shown to be nontoxic (11). The chemical structure of the lipid A moiety of this LPS was determined as the monophosphoryl lipid A (12). DPLA from this source (an analog of toxic DPLA) has been found to be inactive in several biological assays (13) (activation of B lymphocytes (27) and induction of cytokines in macrophages (28). Its chemical structure is shown in Fig. 1A.

We are now able to compare the structure-to-function relationship of a model toxic hexaacyl DPLA with the nontoxic pentaacyl DPLA from R. sphaeroides LPS. The most interesting difference between the two structures is the presence of 3-oxo and unsaturated fatty acids in the R. sphaeroides DPLA. A review article suggested that these fatty acids might contribute toward the lack of toxicity of R. sphaeroides LPS (14). The question we posed was, do these two fatty acids play an important role in the essential nontoxicity of the DPLA?

We prepared the nontoxic DPLA from the LPS of R. sphaeroides, catalytically reduced this preparation in the presence of platinum oxide, and tested it in several key in vitro biological assays. We report that reduction of R. sphaeroides DPLA does not convert it to a biologically active lipid A.

EXPERIMENTAL PROCEDURES

Materials—The following materials were used: HPLC-grade chloroform, methanol, acetonitrile, and isopropyl alcohol (Analab, Inc., North Haven, CT); Dowex 50W-X8, Chelex-100, and Cellex-D (Bio-Rad); silica gel plates (Analtech, Inc., Newark, DE); cytochrome c (type III), superoxide dismutase, bovine erythrocytes, Histopaque (1.077), phorbol myristate acetate, gelatin (type I), and L-glutamine (Sigma); and Hanks' balanced salt solution RPMI 1640 and HEPES buffer (GIBCO).

poly: OHC16, hydroxycdecanoate; OHC17, hydroxytetradecanoate; 3KC16, 3-oxotetradecanoate; 3C16, 3-oxotetradecanoate; Δ1-C16OH, Δ2-tetradecenoyloxytetradecanoate; C16OCH17, tetradecanoyloxytetradecanoate; TNF, tumor necrosis factor; TLC, thin layer chromatography; PMA, phorbol myristate acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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The abbreviations used are: LPS, lipopolysaccharide; DPLA, diphosphoryl lipid A; HPLC, high performance liquid chromatography; OHC16, hydroxycdecanoate; OHC17, hydroxytetradecanoate; 3KC16, 3-oxotetradecanoate; 3C16, 3-oxotetradecanoate; Δ1-C16OH, Δ2-tetradecenoyloxytetradecanoate; C16OCH17, tetradecanoyloxytetradecanoate; TNF, tumor necrosis factor; TLC, thin layer chromatography; PMA, phorbol myristate acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Growth of Bacteria and Preparation of LPS—*R. sphaeroides* ATCC 17023 was grown phototrophically in 2-liter Erlenmeyer flasks in medium 550 as previously described (12). Cells were grown at 26 °C for 12-14 days, concentrated using a Pellicon cassette system (Millipore Corp., Bedford, MA), and harvested by centrifugation. A yield of 850 g of cell paste was obtained from 100 liters of culture.

The cell paste (700 g) was extracted for 1 h, with stirring, in 4 liters of ethanol/n-butyl alcohol (3:1, v/v). This was followed by three 1-h extractions with 4 liters of ethanol and three 1-h extractions with 4 liters of acetone. Finally, the cells were suspended in 2 liters of distilled ether and filtered, yielding 70.4 g of dry, pigment-depleted cells. LPS was extracted from these cells by the phenol/chloroform/petroleum ether method (15) and precipitated as described by Qureshi et al. (7), to yield 640 mg of LPS.

Preparation of DPLA from *R. sphaeroides* LPS—LPS (640 mg) obtained from *R. sphaeroides* was suspended in 0.02 M sodium acetate, pH adjusted to 2.5 with acetic acid, at a concentration of 3 mg/ml. This was incubated for 70 min at 100 °C. The DPLA was recovered as previously described (7).

Crude DPLA (140 mg) was dissolved in chloroform/methanol (2:1, v/v) and applied to a 3.5 × 29-cm DEAE cellulose column (acetate form). The column was washed with 250 ml of chloroform/methanol/water (2:3:1, v/v). DPLA was eluted from the column using a linear gradient of 0.03-0.08 M ammonium acetate in chloroform/methanol/water (2:3:1, v/v). Thirteen-m1 fractions were collected for analysis of total phosphorus (16) (Fig. 2). The peak fractions were analyzed by TLC using Silica Gel H and a solvent system of chloroform/pyridine/88% formic acid/water (10:12:3, v/v). Fractions were pooled according to the profile of the total phosphorus analysis and analytical TLC. The pooled fractions were desalted in a two-phase chloroform/methanol/water system as previously described (6).

The following pooled fractions were obtained (Fig. 2): peak A (fractions 14-19, 11.9 mg), Rf = 0.75 on TLC in the chloroform/pyridine/88% formic acid/water system mentioned above; peak B (fractions 52-61, 11.9 mg), Rf = 0.20; and peak C (fractions 67-87, 28.2 mg), Rf = 0.59. Peak C represented the rontoxic, highly purified pentaacyl DPLA.

Preparation of DPLA from *E. coli* LPS—The hexaacyl DPLA was prepared as previously described (6). Briefly, LPS from *E. coli* D31m4 was treated with 20 mM sodium acetate, pH 4.5, at 100 °C for 10 min. The DPLA was recovered on a BIO-ION Nordic (Uppsala, Sweden) BIN-10K plasma desorption time-of-flight mass spectrometer equipped with a PDP 11/73-based data system. Purified tetramethyl DPLA was dissolved in chloroform/methanol (4:1, v/v) solution and electrosprayed onto a mylar-backed aluminum foil. Positive ion mass spectra were recorded with an accelerating potential of 16 kV for 3 to 9 million primary events, with resolution of 1 ns/channel. H+ and Na+ were used for calibration.

Fast atom bombardment mass spectra were obtained on a Kratos (Manchester, England) MS-50 high-resolution, double-focusing mass spectrometer equipped with an Ion Tech (Teddington, United Kingdom) sledge-field ion gun. Samples were desorbed from the monothioglycerol matrix by a beam of 8 keV Xe atoms. Positive ion spectra were recorded with an accelerating potential at 8 kV over the mass range of 200-350 at a rate of 30 s/decade.

NMR Spectroscopy—Methylated DPLA samples were dissolved in benzene-d6/Me2SO-d6 (9:1) with internal Me4Si as a chemical shift reference. NMR spectra were recorded on a GN-500 spectrometer with a spectral width of 4800 Hz at 500 MHz. One-dimensional spectra were recorded with 8,192 data points and two-dimensional H-H correlation spectra were obtained by a one-pulse sequence 90°-t-90°-t-Acq (with proper phase cycling) allowed for coherence transfer through a double quantum filtered-COSY. Two sets of 384 × 2048 data points were acquired in adjacent blocks of memory, and the data were processed to obtain pure absorptive spectra. Prior to the Fourier transformation, 30° shifted sine bell functions were used in both dimensions and the t1 free induction decay was zero-filled to obtain a final data matrix of 2,048 × 2,048 real points.

**Superoxide Anion Release—**Human bronchoalveolar cells (30% macrophages, >95% viable) were obtained from healthy nonsmoking subjects by bronchoalveolar lavage. These cells were cultured in sterile, 96-well plastic microtiter plates. Cells were suspended in supplemented RPMI 1640 with 2% autologous serum, plated (1 × 10^5 cells/well), and allowed to adhere at 37 °C in a humidified 5% CO₂ atmosphere for 1 h. The supernatant medium and nonadherent cells were aspirated, cells were washed once in medium, and the adherent cells were conditioned overnight in supplemented RPMI 1640 with 10% autologous serum. The cells were then cultured for 48 h at 37 °C in a 5% CO₂ atmosphere in supplemented RPMI 1640 with 2% autologous serum.
Reduced DPLA from *R. sphaeroides*

serum and various concentrations of DPLA. Stock solutions of DPLA in 0.125% triethylamine were diluted and used for the experiment. All experiments were performed under low endotoxin conditions.

PMA-stimulated superoxide anion production by alveolar macrophages was determined as superoxide dismutase-inhibitable reduction of ferricytochrome c by the method of Calhoun et al. (19). A 200-μl final volume contained 0.1% gelatin, 1.2 mg/ml cytochrome c, and PMA (20 ng/ml). Paired reactions were run with superoxide dismutase. Absorbance was measured at 550 nm over a 2-h period. Plates were incubated at 37°C. Kinetic analysis showed that peripheral blood monocyte and alveolar macrophage superoxide anion release increased linearly for at least 2 h. Triethylamine in the culture medium did not affect superoxide anion generation by cultured cells.

**RESULTS**

**Preparation and Preliminary Characterization of DPLA from LPS of *R. sphaeroides*—**The LPS of *R. sphaeroides* was acid hydrolyzed to yield crude DPLA. This was purified by DEAE-cellulose column chromatography to yield several peak fractions (Fig. 2). Peak A represented the monophosphoryl lipid A. Peaks B and C were two forms of DPLA that could be differentiated by TLC using silica gel H and chloroform/pyridine/formic acid/water (10:12:3:1, v/v) but not by the reverse-phase HPLC using a C18-bonded silica cartridge. A pentaacyl DPLA eluted from the cartridge between 15 and 23 min. The set of very small peaks appearing at elution times of 10-15 min were identified as the tetraacyl series of DPLA. The fatty acid analysis of HPLC peak 1 by gas-liquid chromatography of the acid-hydrolyzed and methylated samples (12) revealed that a single molecule of pentaacyl DPLA eluted from the column. The tetramethyl DPLA was never strictly established, we decided to examine its structure by mass spectrometry. The HPLC-purified tetramethyl DPLA peaks (Fig. 3A) were analyzed by plasma desorption mass spectrometry. The HPLC-purified methylated DPLA. A C18-bonded silica cartridge and a linear gradient of isopropanol alcohol in acetonitrile were used. The sample size injected was 1.0 μg and the AUF was set at 0.16. This graph is base line corrected.

**Preparation and Preliminary Characterization of DPLA from LPS of *R. sphaeroides*—**The LPS of *R. sphaeroides* was acid hydrolyzed to yield crude DPLA. This was purified by DEAE-cellulose chromatography to yield several peak fractions (Fig. 2). Peak A represented the monophosphoryl lipid A. Peaks B and C were two forms of DPLA that could be differentiated by TLC using silica gel H and chloroform/pyridine/formic acid/water (10:12:3:1, v/v) but not by the reverse-phase HPLC using a C18-bonded silica cartridge. A pentaacyl DPLA eluted from the cartridge between 15 and 23 min. The set of very small peaks appearing at elution times of 10-15 min were identified as the tetraacyl series of DPLA. The fatty acid analysis of HPLC peak 1 by gas-liquid chromatography of the acid-hydrolyzed and methylated samples (12) revealed that a single molecule of pentaacyl DPLA has two OHC10, one Δ2-C14, one OHC14, and one 3KCH3 (Table 1).

**Mass Spectral Analysis of Tetramethyl DPLA—**Since the structure of the *R. sphaeroides* DPLA was never strictly established, we decided to examine its structure by mass spectrometry. The HPLC-purified tetramethyl DPLA peaks (Fig. 3A) were analyzed by plasma desorption mass spectrometry (Fig. 4). The molecular ions MNa+ for the major peaks 1 and 2 were observed at m/z 1,578 and 1,580, respectively. The loss of PO4CH3 (−108) accompanied by proton transfer gave rise to ions at m/z 1,470 and 1,472, respectively. Additional loss of OHCH3 yielded MNa+ minus OHC10 and PO4CH3 ions at m/z 1,281 and 1,283, respectively. These results showed that the M− for peaks 1 and 2 (Fig. 3A) are 1,555 and 1,557 for components 1 and 2 (Table I).

These results are consistent with the sizes of dimethyl monophosphoryl lipid A established by Qureshi et al. (12), which were 1,447 and 1,449, suggesting the presence of an additional phosphate group (PO4CH3, 108 amu) in the DPLA series. There was no evidence of a pyrophosphate group. A peak at m/z 124 in the negative ion mass spectrum (data not shown) corresponds to the dimethylated phosphate anion PO4(CH3)2 and most likely results from the cleavage of the C-O bond of the sugar 1-phosphate. An analogous peak corresponding to the anion of a pyrophosphate group at this position was not observed.

Fast atom bombardment and plasma desorption mass spec-


**TABLE I**

Comparison of fatty acid distribution of a synthetic DPLA and DPLA from LPS of E. coli and R. sphaeroides

| Lipid A                          | M<sup>+</sup> (CH<sub>3</sub>) | R<sub>1</sub> | R<sub>2</sub> | R<sub>3</sub> | R<sub>4</sub> |
|---------------------------------|--------------------------------|-------------|-------------|-------------|-------------|
| RsDPLA component<sup>a</sup>    | 1,555                          | OHC<sub>10</sub>Δ<sup>2</sup>-C<sub>14</sub>OC<sub>14</sub> | OHC<sub>10</sub>3K<sub>14</sub> |             |             |
| RsDPLA component<sup>b</sup>    | 1,557                          | OHC<sub>10</sub>C<sub>14</sub>OC<sub>14</sub> | OHC<sub>10</sub>3K<sub>14</sub> |             |             |
| Reduced RsDPLA<sup>c</sup>      | 1,599                          | OHC<sub>10</sub>C<sub>14</sub>OC<sub>14</sub> | OHC<sub>10</sub>OH<sub>14</sub> | OH<sub>14</sub>C<sub>14</sub>OC<sub>14</sub> | OH<sub>14</sub>OHC<sub>14</sub> |
| EcDPLA                          | 1,599                          | OHC<sub>10</sub>C<sub>14</sub>OC<sub>14</sub> | OHC<sub>10</sub>OH<sub>14</sub> | OH<sub>14</sub>OC<sub>14</sub> | OH<sub>14</sub>OHC<sub>14</sub> |
| Synthetic DPLA<sup>d</sup> (Ib) |                                 |             |             |             |             |

<sup>a</sup> Reference to Fig. 1 for chemical structures.

<sup>b</sup> These two are the major components of peak C and represent HPLC peaks 1 and 2 (Fig. 3A).

<sup>c</sup> This represents HPLC peak 4 (Fig. 3B) of the catalytically reduced peak C.

<sup>d</sup> EcDPLA is the model toxic hexaacyl DPLA derived from the LPS of E. coli.

<sup>e</sup> The properties of this synthetic product (pentaacyl DPLA) were reported by Rietschel et al. (25).

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**TABLE II**

'H NMR chemical shifts (in ppm from internal Me<sub>4</sub>Si) and vicinal coupling constants (J<sub>H-H</sub>, Hz) for the disaccharide of HPLC purified tetramethyl DPLA from LPS of R. sphaeroides

| H<sup>1</sup>-N-H                        | H<sub>2</sub>, H<sub>3</sub>, H<sub>4</sub>, H<sub>5</sub>, H<sub>6a</sub>, H<sub>6b</sub>, N-H |
|----------------------------------------|--------------------------------------------------|
| (J<sub>H1-H2</sub>)                    | (J<sub>H3-H2</sub>) (J<sub>H4-H2</sub>) (J<sub>H5-H2</sub>) (J<sub>H6a-H2</sub>) (J<sub>H6b-H2</sub>) |
| 5.99                                   | 4.65                                             | 5.61                                             | 3.87                                             | 4.39                                             | 4.07                                           | 8.19                                          |
| (2.8)                                  | (3.8)                                            | (4.4)                                            |                                                   |                                                   |                                               |                                               |
| 5.13                                   | 4.20                                             | 5.69                                             | 4.76                                             | 3.61                                             | 3.93                                           | 4.05                                          | 8.27                                          |
| (8.2)                                  |                                                  |                                                   |                                                   |                                                   |                                               |                                               |                                               |

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**Reduced DPLA from R. sphaeroides**

The abbreviations used are: RsDPLA, R. sphaeroides DPLA; EcDPLA, E. coli DPLA.

shows that H-1 is equatorial and the reducing-end glucosamine is in the α-anomeric configuration (22). Similar results were obtained with HPLC peak 2 (Fig. 3A).

Catalytic Reduction of E. coli and R. sphaeroides DPLA—E. coli DPLA and peak C were reduced with H<sub>2</sub> in the presence of platinum oxide and analyzed for completeness of reaction. Fatty acid analysis of the reduced peak C by gas-liquid chromatography revealed the complete absence of 3K<sub>14</sub> and Δ<sup>2</sup>-C<sub>14</sub> (data not presented). Reverse-phase HPLC of the tetramethyl ester showed a shift in the position of the two major peaks from 16 min for peak 1 and 18 min for peak 2 to a single major peak 4 at 24 min (Fig. 3, A and B).

Peak 4 was analyzed by plasma desorption mass spectrometry (Fig. 4C). It showed a molecular ion M<sup>+</sup>N<sub>a</sub>* at m/z 1,582. Loss of PO<sub>4</sub>C<sub>2</sub>H<sub>2</sub> gave rise to ions at m/z 1,474. Additional loss of OHC<sub>10</sub> yielded M<sup>+</sup>N<sub>a</sub>* minus PO<sub>4</sub>C<sub>2</sub>H<sub>2</sub> and OHC<sub>10</sub> ion, or at m/z 1,285. This showed that the M<sub>a</sub> of peak 4 is 1,559 (Fig. 3B, Table I), indicating that the 3K<sub>14</sub> and Δ<sup>2</sup>-C<sub>14</sub> were converted to OHC<sub>14</sub> and C<sub>14</sub>OH<sub>2</sub> respectively. Catalytic reduction of 3K<sub>14</sub> should yield a racemic acid containing equivalent amounts of Δ<sup>2</sup>- and L-enantiomers.

The platinum oxide/H<sub>2</sub>-treated E. coli DPLA was similarly analyzed by HPLC and found to be identical to the untreated sample (data not presented). As expected, treatment of E. coli DPLA with platinum oxide/H<sub>2</sub> had no effect on the structure as determined by plasma desorption mass spectrometry. These preparations served as the positive controls in the biological assays.

**Effects of R. sphaeroides DPLA on the Priming of PMA-stimulated Superoxide Anion Release by Human Alveolar Macrophages—** Pabst and Johnston (23) first showed that when peritoneal macrophages are exposed to LPS, the cells are primed to respond with increasing oxygen anion (O<sub>2</sub>) production after stimulation with PMA. This secretion of oxygen intermediate was suggested by Nathan (24) to be a biochemical marker of macrophage activation. Since this appears to be an excellent and sensitive model system for LPS activation, we examined the ability of both normal and reduced R. sphaeroides DPLA to prime the PMA-stimulated O<sub>2</sub> release in human alveolar macrophages. When these two DPLAs were tested over a concentration range of 0.01–100 ng/ml, they showed virtually no priming activity (Fig. 5). Thus, the conversion of the 3-oxo group to 3-hydroxy and saturation of the double bond of the fatty acids of R. sphaeroides DPLA did not change its lack of priming activity.

Control samples of normal and platinum oxide/H<sub>2</sub>-treated E. coli DPLA were very effective in priming macrophages (Fig. 5). Activity was observed even at 0.01 ng/ml, and reached a maximum at about 10 ng/ml. This showed that treatment of E. coli DPLA with platinum oxide/H<sub>2</sub> does not affect its ability to prime the macrophages.

**Effect of Reduced R. sphaeroides DPLA on Induction of TNF in Macrophages—** We have already shown that R. sphaeroides DPLA is unable to induce the formation of TNF by RAW 264.7 cells (13). Table III again shows this to be the case. Reduced R. sphaeroides DPLA was also inactive in the induction of TNF. The value of 367 TNF units at 10<sup>5</sup> ng/ml is only 1.8% of the units that the toxic E. coli DPLA showed at the same concentration. The E. coli DPLA and the platinum oxide/H<sub>2</sub>-treated E. coli DPLA were equally effective in inducing TNF by the RAW 264.7 cells. These results showed that the reduction of R. sphaeroides DPLA does not cause it to become active in this assay.

**Activation of 70Z/3 Cells by Reduced R. sphaeroides DPLA—** We have shown that the pentaacyl DPLA from LPS of R. sphaeroides is not able to activate 70Z/3 cells. Tested over a...
range of 0.1–30 µg/ml, the *R. sphaeroides* DPLA is indeed not active (Fig. 6). When the reduced DPLA was tested over the same concentration range, it showed a gradual but small increase in the activation of 70Z/3 cells from about 8.5% at 0.1 µg/ml to 17.5% at 1.0 µg/ml. With increase in concentration beyond this point, there was a gradual decrease to about 10% at 30 µg/ml. We consistently observed this pattern. Thus the *R. sphaeroides* DPLA appears to give a small increase in its ability to activate B lymphocytes. Because of the nonlinearity of this rise, it is difficult to interpret the results. However, it is clear that the reduction of *R. sphaeroides* DPLA does not convert it to an impressively active compound.

**DISCUSSION**

We have developed a simplified two-step procedure for preparing pentaacyl DPLA from the LPS of *R. sphaeroides*. The first step is the acid hydrolysis of LPS with 0.02 M sodium acetate, pH 2.5 (instead of the usual pH 4.5 used with *E. coli* and *Salmonella* LPS (5, 6)), to yield the crude DPLA. The second step is the DEAE-cellulose column fractionation of the hydrolyzed LPS, which effectively separated the unhydrolyzed LPS, monophosphoryl lipid A, and several forms of DPLA. This new procedure was used to prepare the DPLA for this study.

*R. sphaeroides* DPLA was characterized by combined reverse-phase HPLC and mass spectrometry and ¹H NMR spectroscopy. Plasma desorption and fast atom bombardment mass spectrometry were utilized to establish and confirm the structure of the two major HPLC fractions (components 1 and 2) as the tetramethyl DPLA instead of the dimethyl monophosphoryl lipid A, as was done previously (12). The α-anomeric configuration of the reducing-end phosphate was established by ¹H NMR spectroscopy.

This purified nontoxic pentaacyl DPLA (peak C) was catalytically reduced with platinum oxide. The completeness of reduction was confirmed by gas-liquid chromatography of the fatty acids as well as reverse-phase HPLC and plasma desorption mass spectrometry of the reduced DPLA.

The reduced DPLA was then tested for its ability to prime the PMA-stimulated superoxide anion release in human alveolar macrophages, induce TNF production in the RAW 264.7 macrophage cell line, and activate 70Z/3 pre-B cells. We found that reduction of *R. sphaeroides* DPLA did not convert it to an active form in any of these assays. The slight activation of 70Z/3 cells by the reduced *R. sphaeroides* DPLA is not considered significant in this study. Both the *R. sphae-
Reduced DPLA and the reduced *R. sphaeroides* DPLA completely blocked the ability of the *E. coli* D31m4 LPS to prime the PMA-stimulated superoxide anion release in human alveolar macrophages. *E. coli* D31m4 LPS at 0.1 ng/ml showed an activity of 9.95 ± 1.24 nmol of superoxide anion/10⁶ cells/h. *R. sphaeroides* DPLA (100 ng/ml) plus *E. coli* LPS (0.1 ng/ml) and reduced *R. sphaeroides* DPLA (100 ng/ml) plus *E. coli* LPS (0.1 ng/ml) showed an activity of 1.90 ± 0.10 and 1.96 ± 1.1 nmol of superoxide anion/10⁶ cells/h, respectively. Control was 2.18 ± 0.81 nmol. These results showed that the 3-oxo group and unsaturation of the fatty acids of *R. sphaeroides* DPLA are not the factors that made the compound inactive and nontoxic.

When one compares the structures of the model toxic hexaacyl DPLA (Fig. 1B) with the nontoxic pentaacyl DPLA (Fig. 1A), the following differences are noted: (i) the toxic DPLA contains six fatty acids, whereas the nontoxic DPLA has only five; (ii) the nontoxic DPLA has much shorter fatty acids (OHC₃₀) at R₁ and R₃ than the toxic DPLA; (iii) the nontoxic DPLA has greater variation in the sizes of fatty acids than the toxic DPLA; (iv) the nontoxic DPLA has an unsaturated fatty acid in acyloxyacyl linkage at R₂; (v) the nontoxic DPLA has a 3KC₁₄ at R₅. Any of these factors could account for the difference in their biological activities. However, the results of this study strongly suggest that the lack of biological activities of *R. sphaeroides* DPLA is not due to the last two factors.

Rietschel *et al.* (25) showed that the synthetic pentaacyl DPLA called Ib-isomer (Table I) is less toxic (5- to 10-fold in *in vivo* assays) than the model hexaacyl DPLA (*E. coli* type). However, very similar activities were observed for the hexa- and pentaacyl DPLA in the B cell mitogenic assay. It is interesting to note that this Ib-isomer has structural features in common with our reduced *R. sphaeroides* DPLA (compare reduced RsDPLA with synthetic DPLA (Ib-isomer) in Table I). However, two major differences between the reduced *R. sphaeroides* DPLA and Ib-isomer exist in the composition of R₁ and R₃. The R₁ and R₃ of reduced *R. sphaeroides* DPLA are OHC₁₆, whereas those of Ib-isomer are OHC₁₈. Similar differences exist when we compare DPLA from *R. sphaeroides* and *E. coli*, as shown by the molecular models in Fig. 7. These structural representations emphasize the importance of the size of the hydroxy fatty acids at R₁ and R₃ of the DPLA in determining biological activity. We propose that when R₁ and R₃ have hydroxy fatty acids equal to or less than C₁₀ and the total fatty acid content is five, the DPLA will be nontoxic and biologically inactive. When R₁ and R₃ are OHC₁₄ and the total fatty acid content is six, the DPLA should be toxic and biologically active. This raises an interesting question: what is the biological property of a DPLA when R₁ and R₃ are both OHC₁₆? For such a study, we plan to isolate and purify the DPLA from LPS of *Neisseria gonorrhoeae* (26) and compare its biological activities with the model toxic DPLA from *E. coli* LPS and the reduced pentaacyl DPLA from *R. sphaeroides* LPS.
If the 3-oxo group in the lipid A moiety is not making the LPS nontoxic, one might wonder what its physiological role might be. It is established that the hexaacyl lipid A is more active than the pentaacyl lipid A (25). The 3-oxo group (but not the 3-hydroxy group) at R4 would prevent the acylation of that fatty acid (to form the acyloxyacyl group) to increase the fatty acid content to six and thus yield a more active and toxic LPS.

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