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Agonistic and Antagonistic Activities of Bacterially Derived *Rhodobacter sphaeroides* Lipid A: Comparison with Activities of Synthetic Material of the Proposed Structure and Analogs

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Lipid A from the photosynthetic bacterium *Rhodobacter sphaeroides* (RSLA) has been previously shown to antagonize many of the effects of endotoxins from more pathogenic gram-negative bacteria. We have reported on the synthesis of the proposed structure of RSLA and determined that bacterially derived RSLA is not identical to its proposed structure (W. J. Christ, P. D. McGuinness, O. Asano, Y. Wang, M. A. Mullarkey, M. Perez, L. D. Hawkins, T. A. Blythe, G. R. Dubuc, and A. L. Robidoux, J. Am. Chem. Soc. 116:3637–3638, 1994). Here we report results of analyzing the antagonistic and agonistic activities of bacterially derived RSLA in comparison with the activities of chemically synthesized material of the proposed structure of RSLA and analogs. Results indicated that all compounds were approximately equally potent at inhibiting endotoxin-induced release of tumor necrosis factor alpha from human monocytes and human whole blood as well as endotoxin-induced generation of nitric oxide in murine macrophages. In addition, all compounds were of equivalent potencies at inhibiting the binding of ^{125}I-labelled lipopolysaccharide derivatized with 2-(*p*-azidosalicylamido) ethyl-1-3′-dithiopropionate to murine macrophages. Higher concentrations of bacterially derived RSLA (10 to 100 μM) were agonistic in human and murine assays. In gamma interferon-treated murine macrophages, agonism was exhibited at concentrations as low as 100 nM. In contrast, all synthetic materials were either dramatically less agonistic or devoid of agonistic activity when tested at concentrations as high as 100 μM. It is possible either that bacterially derived RSLA contains a small amount of a highly agonistic impurity or that the agonistic activity of RSLA is intrinsic to its molecular structure. In either case, these biological results support our previous report concluding that biologically derived RSLA is not identical to synthetic material of its proposed structure.

Lipopolysaccharide (LPS or endotoxin) is a major structural component of the outer membranes of gram-negative bacteria. During bacterial infections, LPS shed from pathogenic bacteria can trigger a broad range of deleterious responses from LPS-sensitive cells such as macrophages, monocytes, and neutrophils (22, 27). The portion of the LPS molecule that possesses most or all of the attributed toxic activity has been shown to be the lipid A moiety (6). In contrast, the LPSs of the gram-negative photosynthetic bacteria *Rhodobacter sphaeroides* (25) and *Rhodobacter capsulatus* (18) as well as deacylated LPS from *Neisseria* spp. (3) are relatively nontoxic. Both *R. sphaeroides* LPS (RsLPS) and lipid A (RSLA) are relatively nonagonistic and can effectively block some of the in vitro and in vivo effects of agonistic LPSs from infectious gram-negative bacteria such as *Escherichia coli* (8, 14, 18, 21, 25; reviewed in reference 20). In in vitro assay systems, lipid A's from *R. sphaeroides* and *R. capsulatus* can antagonize many of the known effects of endotoxin, including binding to murine and human cells (17, 18), activation of human promonomyelocytic cells (7), stimulation of cytokine release from murine macrophages (8), and expression of LPS-inducible genes (21). Similarly, RSLA has been shown to inhibit LPS-induced release of tumor necrosis factor alpha (TNF-α) and LPS-induced lethality in mice (24, 36).

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A proposed structure of RSLA (Fig. 1) has been reported (23). This molecule differs from the more agonistic lipid A's (e.g., *E. coli* lipid A) by (i) presence of a keto (instead of hydroxy) group at the 3 position of the myristate attached to the 2 position of the A ring, (ii) a *cis* or *trans* unsaturated acyloxyacyl side chain attached to position 2′ on the B ring, (iii) fewer fatty acid substituents (only one acyloxyacyl), and (iv) shorter chain lengths. Verification of the molecular structure through characterization of RSLA made by synthetic organic procedures has shown that the structure of bacterially derived RSLA is different from that which has been previously reported (1).

As shown in Fig. 1, the synthetic material of the proposed structure (herein referred to as 2′ *cis* LA) contains a *cis* Δ^7 unsaturated acyloxyacyl on the 2′ carbon of the B ring glucosamine and all chiral side chains are of the R configuration (initial references contained no stereochemistry, and subsequent references assumed that the side chains are in the R configuration in the natural product). Two analogs also described (1) and tested here are *trans* Δ^7 unsaturated acyloxyacyl (2′ *trans* LA) and *cis* Δ^7 unsaturated acyloxyacyl on position 2 of the A ring glucosamine (2 *cis* LA).

To compare the biological activities of synthetic lipid A analogs with that of bacterially derived RSLA, we tested the agonistic and antagonistic activities of these compounds in three in vitro assays which measure the stimulation and/or inhibition of cellular mediator release (TNF-α and nitric oxide) from primary human monocytes, human whole blood cultures, and murine macrophage cell lines. In addition, the abil-

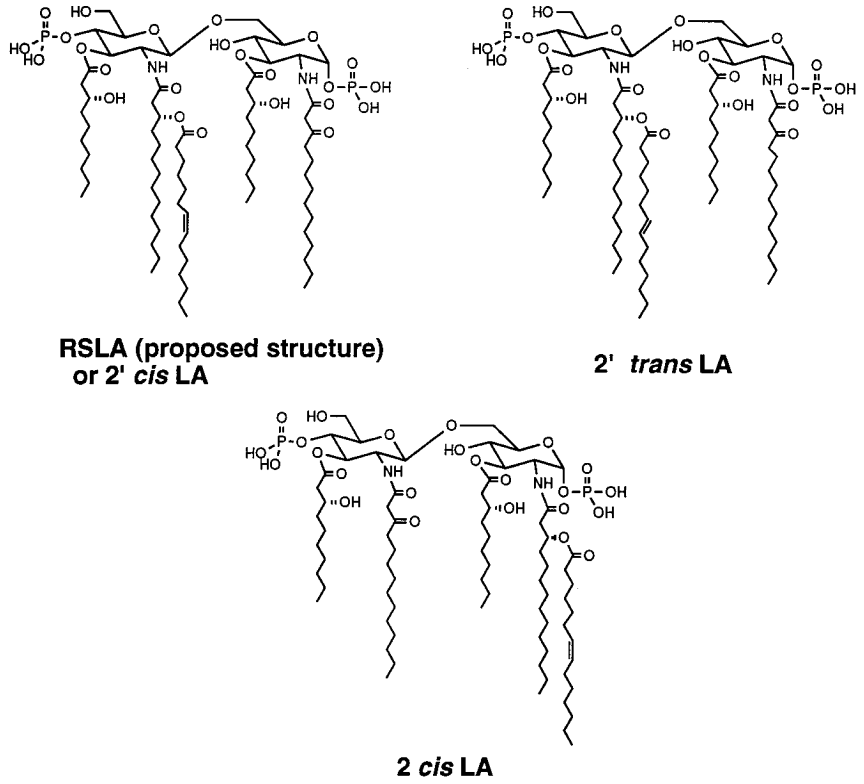

FIG. 1. Structures of 2′ *cis* LA (proposed structure of bacterially derived RSLA), 2 *cis* LA, and 2′ *trans* LA. The structural elucidation of bacterially derived RSLA has been reported previously (23), but the configurations of the C-3 substituents of the fatty acid side chains were not assigned in the natural product.

ity to block LPS binding to the cell surface was examined. Although all of the compounds tested were approximately equivalent antagonists, bacterially derived RSLA was also a uniquely potent inducer of TNF-α and nitric oxide release compared with the synthetic analogs.

MATERIALS AND METHODS

Materials. LPS derivatized with 2-(*p*-azidosalicylamido) ethyl-1-3′-dithiopropionate (ASD-LPS) was radiolabelled with ^{125}I by using the procedures of Wollenweber and Morrison (33). *E. coli* LPS (serotype O111:B4; List Biological Laboratories, Campbell, Calif.) was used for derivatization and radiolabelling. Recombinant murine gamma interferon was obtained from Genzyme Corp., Cambridge, Mass.

Bacterially derived RsLPS and RSLA were purchased from Advanced Medical Research, Madison, Wis., and used without further purification. Stock solutions of RSLA were prepared by solubilization in 0.9% saline at 1 mM concentration, and the RsLPS was reconstituted in sterile water at 1 mg/ml. The sodium salts of 2′ *cis* LA, 2 *cis* LA, and 2′ *trans* LA (all greater than 80% pure, as estimated by high-pressure liquid chromatography [HPLC]) were synthesized as described previously (1) and solubilized in sterile water at 1 mM. In some cases, these stock solutions were stored as frozen aliquots. A single freeze-thaw cycle appeared to have no effect on agonistic or antagonistic activity. All compounds were sonicated in an ultrasonicator (VW-380; Heat Systems-Ultrasonics Inc., Farmingdale, N.Y.) for 1 to 2 min immediately before use. Serial dilutions were made in Ca^{2+}-Mg^{2+}-free Hanks balanced salt solution (Sigma Chemical Co.). *E. coli* LPS (serotype O111:B4; Sigma) was solubilized in sterile distilled water at 100 μg/ml or 1 mg/ml and then stored as aliquots at −20°C.

When possible, experiments were performed a minimum of three times in triplicate. However, one synthetic material (2 *cis* LA) was available only in limited quantities, and so experiments were done twice in duplicate or triplicate. In all cases, synthetic materials were tested along with bacterially derived RSLA in the same assay. Two different lots of bacterially derived RSLA were tested, with comparable results.

Analysis of LPS-induced release of TNF-α from human monocytes. Human whole blood was obtained aseptically from normal volunteers (18 to 51 years old; 110 to 230 lb [ca. 50 to 104 kg]) and transferred to sterile tubes containing heparin (10 U/ml of blood; LyphoMed Inc., Rosemont, Ill.). Human monocytes were isolated from blood by the Leuco Prep system for mononuclear cell isolation (Becton Dickinson, Lincoln Park, N.J.). The harvested monocytes were suspended in serum-free RPMI 1640 and washed twice in this medium with centrifugation at 600 × *g* for 5 min at 4°C. After the final wash, the cells were resuspended in complete assay medium (RPMI 1640 supplemented with 2 mM L-glutamine, penicillin [80 U], and streptomycin [100 μg/ml] [JRH Biosciences, Lenexa, Kans.] plus 10% heat-inactivated human AB serum [Whittaker Bioproducts Inc., Walkersville, Md.]) and then plated onto 48-well plastic tissue culture plates (Costar) at 1.2×10^6 to 1.5×10^6 cells per well (0.5 ml). After a 3-h incubation at 37°C in 5% CO_2, nonadherent cells were removed by two gentle washes with serum-free RPMI 1640. Isolated monocytes in each well were first overlaid with complete assay medium plus human AB serum (1%, final concentration), and then 50 μl of a 10× solution of antagonist and 50 μl of a 10× LPS solution (10 ng/ml, final concentration) were added. Cells were incubated for 3 h at 37°C in 5% CO_2, and then supernatants were removed and frozen at −80°C. After thawing, samples were centrifuged (2,000 × *g* for 5 min at 4°C) and assayed for TNF-α by enzyme-linked immunosorbent assay (ELISA) (R & D Systems, Minneapolis, Minn., or Genzyme).

TNF-α assays with human whole blood. Human blood was collected as described above except that the heparinized blood was added directly to 48-well microtiter plates (400 μl per well). The indicated concentrations of antagonists were added, followed by LPS (10 ng/ml, final concentration), for a total of 500 μl per well (final concentration of whole blood was 80%). After a 3-h incubation as described above, plates were centrifuged at 1,000 × *g* for 10 min at 4°C, and plasma was drawn off and frozen at −80°C. Plasma samples were analyzed for TNF-α as described above. All compounds were tested at least twice in triplicate.

Induction of nitric oxide in murine macrophages. RAW 264.7 cells (American Type Culture Collection) were cultured in Ham's F12–10% fetal calf serum (JRH Biosciences) containing penicillin (80 U/ml), streptomycin (100 μg/ml) and 2 mM L-glutamine. For use in the assay, cells were seeded into 96-well plates (2×10^5 cells per well) and then allowed to adhere for 3 to 20 h. The monolayers were washed once with serum-free Dulbecco modified Eagle medium (GIBCO), and then 200 μl of the same medium containing antibiotics, L-glutamine, and 10% fetal calf serum was overlaid. Antagonists and agonists were added from 10× concentrates (25 μl of each). In some cases, the indicated concentration of murine gamma interferon was added prior to inhibitor and/or LPS. After 24 h, nitrite was quantitated by adding 100 μl of Greiss reagent as described previously (30) to 100 μl of culture supernatant and then measuring A_{540} on an ELISA microplate reader (Titertek).

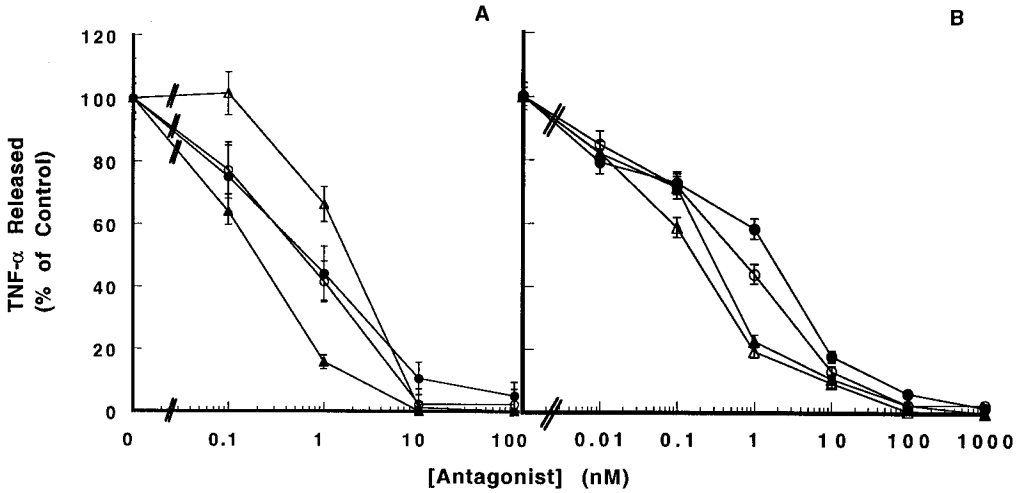

FIG. 2. Inhibition of LPS-induced TNF-α release in human whole blood and in human monocytes. Whole blood (A) or human monocytes (B) were incubated with LPS (10 ng/ml) plus the indicated amount of bacterially derived RSLA (○), 2′ *cis* LA (●), 2 *cis* LA (△), or 2′ *trans* LA (▲). After 3 h, plasma or cell culture medium was obtained and assayed for TNF-α as described in Materials and Methods. When LPS was omitted, no measurable TNF-α was released. Data are representative of three experiments done in triplicate except for 2 *cis* LA, which was assayed twice in triplicate.

Measurement of LPS binding to murine macrophages. RAW 264.7 cells were cultured in Ham's F12 medium as described above. Cells were seeded into 24-well plates (5×10^5 cells per well at plating) and incubated overnight. They were then washed once in serum-free RPMI 1640, and 200 μl of RPMI 1640 containing 0.625% serum was overlaid. Then 25 μl of inhibitor and 25 μl of [^{125}I]ASD-LPS (~25 ng at ~500 μCi/μg) were added to each well. After incubation for 90 min at 37°C, the cells were washed three times with 1 ml of wash buffer containing 50 mM Tris buffer (pH 7.4), 150 mM NaCl, and 2 mg of bovine serum albumin per ml. One milliliter of 0.1 N NaOH was added to each well to solubilize the contents, and then 0.95 ml of this solution was analyzed for radioactivity. To determine if cellular uptake of LPS was involved in our measurements of binding, some assays were done in the presence of 0.2% azide. Under these conditions, no significant differences were observed in time dependence or amount of binding, indicating that there was no azide-sensitive uptake. In addition, experiments utilizing fluorescently labelled LPS with CD14-transfected CHO cells (9) that demonstrated binding equivalent to that in RAW cells indicated that no trypsin-insensitive component of uptake was measurable. However, we have not taken rigorous steps to rule out uptake as a minor component of our binding measurements.

RESULTS

Bacterially derived and synthetic lipid A's inhibit LPS-induced TNF-α release in human whole blood and purified monocytes. As a measure of cellular response to *E. coli* LPS, we quantitated the release of TNF-α by cells in human whole blood and by purified monocytes in primary culture. Without LPS, incubation of fresh whole blood for 3 h at 37°C resulted in no measurable release of TNF-α into the plasma fraction. In survey experiments with whole blood, it was determined that optimal release of TNF-α occurred after 3 h of incubation with LPS, and this response was highly dose dependent from 1 to 10 ng of LPS per ml. Under these conditions, 10 ng of LPS per ml induced an average TNF-α release of 2,875 ± 233 pg/ml (mean ± standard deviation; $n = 11$). RsLPS did not induce measurable TNF-α release when tested at up to 10 μg/ml. Synthetic RSLA and analogs were not measureably agonistic, while bacterially derived RSLA demonstrated agonism at relatively high concentrations (discussed below).

As shown in Fig. 2A and in Table 1, synthetic lipid A's and bacterially derived RSLA potently inhibited LPS-induced TNF-α release in whole blood. Complete inhibition occurred at 10 nM or greater for all compounds when tested against 10 ng of LPS per ml, with the bacterially derived material demonstrating a 50% inhibitory concentration (IC_{50}) value of 0.56 nM. Synthetic RSLA (2′ *cis* LA) showed similar activity (IC_{50} = 0.70 nM), while 2 *cis* LA was slightly less active, with an IC_{50} of 1.32 nM. The 2′ *trans* LA analog was somewhat more active, with an IC_{50} of 0.16 nM.

Similar results were obtained in experiments using purified monocytes. In seven assays, similar responses of cells to 10 ng of LPS per ml were observed, with TNF-α release averaging 3,120 ± 318 pg/ml. Figure 2B and Table 1 demonstrate the antagonistic activities of lipid A's in this monocyte assay; 2′ *trans* LA was slightly more active (IC_{50} = 0.88 nM) than either bacterially derived RSLA (IC_{50} = 1.3 nM), 2′ *cis* LA (IC_{50} = 4.4 nM), or 2 *cis* LA (IC_{50} = 4.3 nM).

LPS-induced nitric oxide synthesis in mouse macrophages and inhibition by lipid A's. Nitric oxide is another cellular mediator produced by endotoxin stimulation of macrophages. Nitric oxide synthase, the enzyme that produces NO, is induced both directly by LPS and indirectly by a variety of cytokines also produced in response to LPS (10, 11, 29). Nitrite, which is quantitated here, is a stable decomposition product of NO. LPS dose dependently stimulated nitrite production in RAW 264.7 cells (19, 32) (data not shown). At LPS concentrations of between 0.5 and 5 ng/ml, stimulation was sharply dependent on the LPS concentration, and at greater than 5 ng of LPS per ml, smaller increases in stimulation of NO release

TABLE 1. Antagonistic activities of bacterially derived RSLA and synthetic lipid A's

Compound	IC_{50}[a] (nM; avg ± SEM)	
	Human whole blood[b]	Human monocytes[c]
Bacterially derived RSLA	0.56 ± 0.24	1.3 ± 0.85
2′ *cis* LA	0.74 ± 0.27	4.4 ± 2
2 *cis* LA	1.32 ± 0.43	4.3 ± 4.17
2′ *trans* LA	0.16 ± 0.11	0.88 ± 0.61

[a] IC_{50} for inhibition of LPS-induced release of TNF-α (LPS = 10 ng/ml).
[b] Average obtained from triplicate determinations for three or more assays for bacterially derived RSLA, 2′ *cis* LA, and 2 *cis* LA, and for two assays for the 2′ *trans* analog.
[c] Average from three assays in triplicate for bacterially derived RSLA and 2′ *cis* LA and for two assays in triplicate for 2′ *trans* LA and 2 *cis* LA.

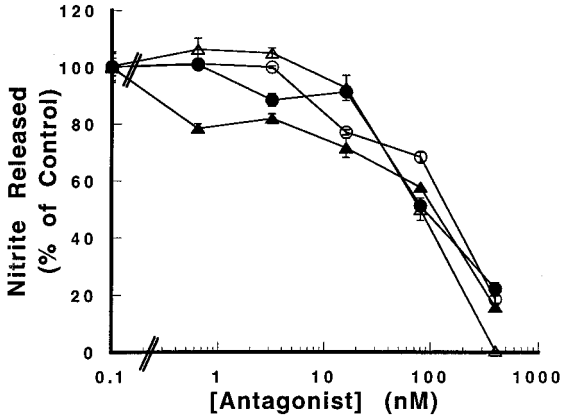

FIG. 3. Inhibition of LPS-stimulated induction of nitric oxide synthesis by bacterially derived RSLA and synthetic lipid A's. RAW 264.7 cells were cultured in the presence of 10 ng of *E. coli* LPS per ml plus the indicated amount of bacterially derived RSLA (○), 2′ *cis* LA (●), 2 *cis* LA (▲), or 2′ *trans* LA (△). After 22 to 24 h, release of nitrite was assayed as described in Materials and Methods. Data are representative of one experiment done three times in triplicate.

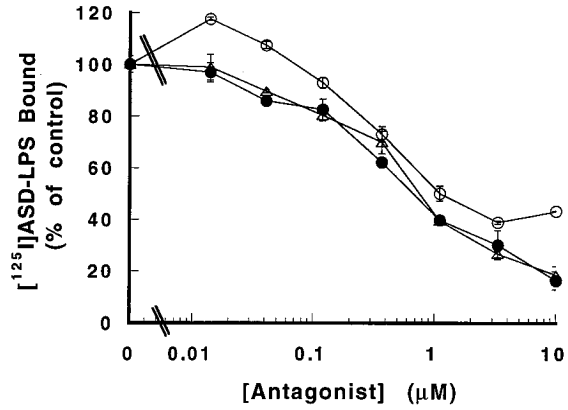

FIG. 4. Inhibition of binding of [^{125}I]ASD-LPS by bacterially derived RSLA and synthetic lipid A's. RAW 264.7 cells were incubated for 90 min with 130,000 to 180,000 cpm of [^{125}I]ASD-LPS plus the indicated concentration of bacterially derived RSLA (○), 2′ *cis* LA (●), or 2 *cis* LA (△) or 1 mg of *E. coli* LPS per ml (nonspecific binding). Binding was determined as described in Materials and Methods. Data are representative of three assays with total binding varying from 2,400 cpm (78% specific binding) to 7,180 cpm (62% specific binding).

were seen as a function of LPS concentration. RsLPS was less active than *E. coli* LPS and required approximately 100-fold-higher concentrations to generate comparable levels of nitrite (data not shown).

All three synthetic lipid A's inhibited induction of NO synthesis by *E. coli* LPS, and as shown in a representative assay in Fig. 3, all were approximately equal in potency. In this series of assays, bacterially derived RSLA was found to have an IC_{50} of 77 ± 26 nM, 2′ *cis* LA had an IC_{50} of 48 ± 16 nM, and both 2 *cis* LA and 2′ *trans* LA exhibited IC_{50}s of between 42 and 55 nM.

Antagonism of [^{125}I]ASD-LPS binding to murine macrophages. Preliminary assays measuring equilibrium binding of [^{125}I]ASD-LPS to RAW 267.4 cells indicated that optimal binding occurred with ~100 ng of [^{125}I]ASD-LPS per ml in the presence of 0.5% fetal calf serum. Under these conditions, specific binding (binding inhibited by 1 mg of unlabelled LPS per ml) was 60 to 80% of total binding. The IC_{50}s for *E. coli* and RsLPS were both between 0.5 and 1 μg/ml. Bacterially derived RSLA demonstrated an IC_{50} of 1.2 ± 0.2 μM (Fig. 4), and synthetic molecules were of approximately equal potencies (IC_{50}s were 1.75 ± 1 μM for 2′ *cis* LA and 1.7 ± 1 μM for 2 *cis* LA). Similar results were obtained when antagonism of LPS binding was measured in human CD14-transfected CHO cells (data not shown).

Agonistic activities of Lipid A's and analogs. In certain assays described above, bacterially derived RSLA and RsLPS demonstrated agonistic activity at high concentrations. Studies examining this agonistic activity are summarized in Table 2. Both RsLPS and RSLA were less potent than 10 ng of *E. coli* LPS per ml but clearly induced an agonistic response in some systems. While RsLPS was only about 10-fold less active than *E. coli* LPS in murine macrophages, it was considerably less active than *E. coli* LPS in human monocytes, even when used at concentrations as high as 1 μg/ml. In whole blood, RsLPS was more than 1,000-fold less potent than *E. coli* LPS at inducing TNF-α release. When tested at 160 μg/ml (100 μM), bacterially derived RSLA induced release of TNF-α or NO in all three systems, demonstrating 27 to 35% of the activity of *E. coli* LPS. In comparison, agonistic activities of 2 *cis* LA (the synthetic material of the proposed structure of RSLA) and 2′ *trans* LA were immeasurable (less than 5% of the activity of LPS) at 160 μg/ml (100 μM). Finally, when used at 160 μg/ml, 2 *cis* LA exhibited less than 10% of the activity of 10 ng of LPS per ml. It should be noted that the greater variability in the calculations of agonistic activities for induction of TNF-α and NO by 2′ *cis* LA and 2′ *trans* LA were due to measurements made at the limits of detection, and the inherent error in making these measurements is reflected in the observed measurement errors (standard errors of the means).

Effect of gamma interferon treatment on antagonistic and agonistic activities in murine macrophages. As previously described (4, 5, 11), murine macrophages can be primed for LPS activation by addition of gamma interferon at nonagonistic concentrations. In our assays, addition of low concentrations of gamma interferon (2 U/ml) resulted in a twofold increase in the agonistic activity of 10 ng of LPS per ml (Fig. 5). At this concentration of gamma interferon, 2′ *cis* LA demonstrated slightly weaker antagonistic activity against LPS (the IC_{50} was increased approximately sixfold; Fig. 6A) while bacterially derived RSLA exhibited less than 50% inhibition even when used at concentrations as high as 10 μM. When the gamma interferon concentration was further increased to 10 U/ml, 2′ *cis* LA was weakly effective (48% inhibition at 1 μM), while bacterially derived RSLA lost all antagonistic activity (data not shown).

The addition of gamma interferon provides a more sensitive method for testing agonistic activity. For example, as shown in Fig. 5, gamma interferon alone at concentrations below 10 U/ml demonstrated little or no activation. However, cells treated with 5 U of gamma interferon per ml responded vigorously to 1 ng of *E. coli* LPS per ml, with an approximately 2.6-fold increase in response over control (LPS alone) levels. Addition of as little as 1 U of gamma interferon per ml resulted in a greater than 10-fold decrease in the threshold response to LPS, from approximately 1 ng to less than 0.1 ng of LPS per ml (data not shown).

When added along with 5 U of gamma interferon per ml, bacterially derived RSLA demonstrated agonistic activity at concentrations ranging from 0.1 to 100 μM (Fig. 6B). In the presence of 5 U of gamma interferon per ml, the level of nitric oxide released in response to stimulation by 10 μM bacterially derived RSLA was approximately equal to that seen with 1 ng of *E. coli* LPS per ml (Fig. 5). Again, the synthetic materials

TABLE 2. Agonistic activities of bacterially derived RSLA and synthetic lipid A's[a]

Compound	Test system	Concn tested (μg/ml)[b]	TNF-α released (pg/ml)	NO released (μM)[c]	Stimulation (% of LPS control)[d]
RsLPS	Whole blood	10	None		0
	Monocytes	1	487		20
		0.1	14		0.6
	RAW cells	1		21.8 ± 0.32	100
		0.1		13.9 ± 0.08	71
Bacterially derived RSLA	Whole blood	160	825 ± 150		35 ± 9
	Monocytes	160	663 ± 270		27.5 ± 12
		16	27 ± 41		1.2 ± 1.8
	RAW cells	160		4.6 ± 0.37	29 ± 9
2′ *cis* LA	Whole blood	160	14 ± 56		1 ± 2
	Monocytes	160	0.65 ± 4.8		.03 ± 0.15
	RAW cells	160		0.52 ± 0.27	1.7 ± 1.6
2 *cis* LA	Whole blood	160	33 ± 33		0.15 ± 1.95
	Monocytes	160	125 ± 80		9.5 ± 5.5
2′ *trans* LA	Whole blood	160	0		0
	Monocytes	160	5.2 ± 3.6		0.35 ± .05
	RAW cells	160		0.3 ± 0.15	3.4 ± 1

[a] Measured as described in Materials and Methods.
[b] 160 μg/ml = 100 μM for RSLA and analogs.
[c] Measured as nitrite.
[d] Calculated from values measured in the same assays as agonist analysis. LPS control is agonistic activity of *E. coli* LPS (10 ng/ml).

were only very weakly agonistic even under these rigorous assay conditions when tested at concentrations of up to 100 μM (Fig. 6B).

Gamma interferon was also used to test for specificity of antagonism by *cis* 2′ LA. As demonstrated in Fig. 5, 20 U of gamma interferon per ml alone can induce synthesis of NO (11, 19). Nitric oxide release induced by gamma interferon alone was inhibited by less than 10% by *cis* 2′ LA at concentrations of up to 100 μM (data not shown), indicating that the antagonistic activity of *cis* 2′ LA was specific for LPS-induced cellular activation.

DISCUSSION

We have previously reported that comparison of bacterially derived RSLA with synthetic material of the proposed structure of RSLA indicated that there are some differences between the bacterially derived material and its proposed structure. Because of the potentially broad interest in the use of RSLA as an LPS antagonist, it became important to explore these differences. The measurement of cellular activation can be performed at several levels by quantitation of a variety of cytokines at both the transcriptional and translational levels (21, 34, 35). From these studies, it can be concluded that regulation of cytokine synthesis, processing, and release involve complex pathways that function under a variety of control mechanisms that can be altered at one or more levels. The goal of our research program is to synthesize compounds that specifically inhibit endotoxin activation of cells, resulting in decreased levels of circulating cytokines and cellular mediators in vivo. For this reason, we have chosen quantitation of TNF-α and NO release as indicators of antagonism of any (or all) steps that lead to their induction. Other results from this laboratory indicate that dose-dependent antagonism of LPS-induced release of TNF-α by lipid A analogs coincides with inhibition of release of interleukins 1, 6, and 8 in our whole blood assays (unpublished results) and inhibition of inducible nitric oxide synthase in RAW cells (32). On the basis of these studies, it is likely that our measurements of TNF-α and NO represent stimulation and/or inhibition of cellular activation.

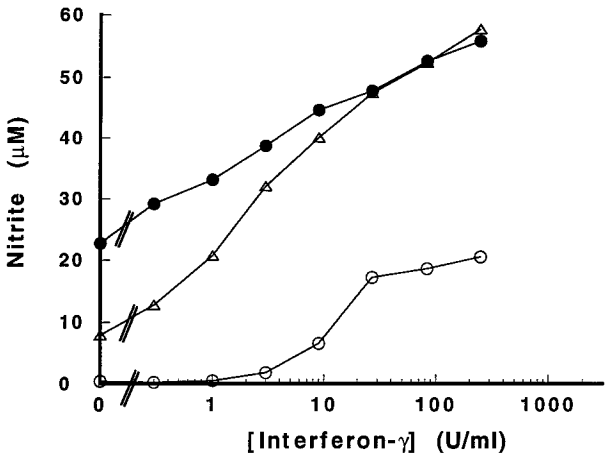

FIG. 5. Activation of murine macrophages by combinations of gamma interferon and LPS. RAW 264.7 cells were cultured in the indicated concentration of gamma interferon (interferon-γ) alone (○) or in the presence of *E. coli* LPS at 1 (●) or 10 (△) ng/ml. After 22 to 24 h, release of nitrite was assayed as described in the legend to Fig. 3.

When tested for the ability to inhibit LPS induction of TNF-α release from whole blood and monocytes (Fig. 2) and LPS-induced generation of NO from murine macrophages (Fig. 3), the natural and synthetic materials exhibited little difference in potency. These results indicated that structural differences between the natural product and the three synthetic analogs were of trivial importance in regard to antagonistic potency.

This lack of major difference in activity may be reflected in the potential of these compounds to block cell surface binding of LPS as analyzed in vitro by using both RAW 264.7 cells and CHO cells transfected with the human CD14 receptor gene (9). All of these compounds demonstrated approximately equivalent activities in these experiments (Fig. 4). However, it is not yet clear whether inhibition of binding can be strictly

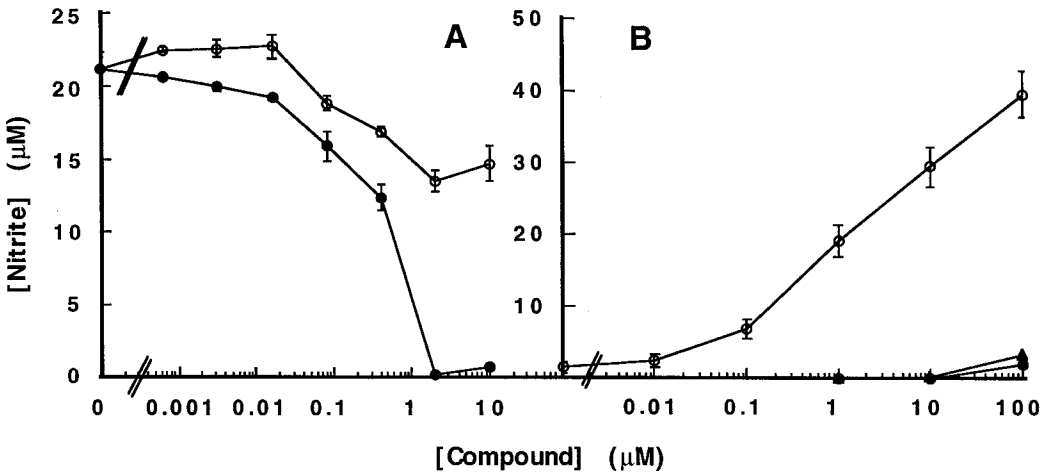

FIG. 6. Effects of gamma interferon on antagonistic and agonistic activities of bacterially derived RSLA and lipid A's. (A) Antagonistic activity in RAW 264.7 cells cultured in 2 U of gamma interferon per ml, 10 ng of LPS per ml, and the indicated concentration of bacterially derived RSLA (○) or 2′ *cis* LA (●). (B) Agonistic activity in RAW 264.7 cells after incubation in the presence of 5 U of gamma interferon per ml plus the indicated concentration of bacterially derived RSLA (○), 2′ *cis* LA (●), or 2 *cis* LA (▲). After 24 h, release of nitrite was assayed as described in Materials and Methods. Data are for a representative experiment done three times in triplicate. Standard deviations of values from the agonism assay were less than 10% for synthetic compounds.

correlated to antagonism of LPS-induced release of cellular mediators. A report by Kitchens et al. (15) argues that these two observations may be unrelated and that inhibition of cellular activation can occur without the inhibition of LPS binding to the cell surface. However, a report by Heine et al. (12) suggests that PE-4, a phosphonooxyethyl analog of compound 406, competitively antagonized both LPS activation and binding at a constant ratio of agonist to antagonist. In our case, inhibition of LPS activity and binding demonstrated two differences. Antagonism of activity occurred at low nanomolar concentrations and was independent of serum concentration (compare panels A and B in Fig. 2). In contrast, inhibition of binding required nearly 1,000-fold more antagonist (approximately 1 μM; Fig. 4), and the ratio of agonist to antagonist required for effective inhibition was highly dependent on the serum concentration (data not shown). Although binding assays and antagonism assays were carried out with different concentrations of LPS (10 ng/ml was used in activation studies, while ~100 ng/ml was used in binding assays), other activation experiments indicated that when the concentration of LPS was increased to 100 ng/ml, only about 20-fold more antagonist was required for equivalent inhibition (data not shown). Measurable inhibition of binding was not observed at concentrations of antagonist that completely inhibit cellular activation. From these results, it is likely that antagonistic lipid A's inhibit at a step subsequent to the binding of an agonist to the CD14 receptor. This finding is in agreement with the conclusions of Kitchens et al. (15). To facilitate the design of novel LPS antagonists, it would be clearly beneficial to understand the structure-activity relationships between inhibition of binding and inhibition of cellular activation. However, such a detailed structure analysis awaits the development of compounds that exhibit a wider range of activities.

As a therapeutic entity, an antagonist should demonstrate no agonistic activity under any of the pathological conditions in which it may be administered. In all cases, synthetic material demonstrated little or no agonistic activity whereas the bacterially derived material was clearly agonistic. Although only weak agonistic activity of bacterially derived RSLA was measurable in human whole blood and purified monocytes, further analysis in murine macrophages primed with gamma interferon revealed potent agonistic activity (Fig. 6B). This observation suggests that cells primed with gamma interferon may provide an extremely sensitive system with which to examine agonistic activity. This synergistic effect of gamma interferon and LPS or other agonistic compounds has been reported to occur in mice (5, 11), in murine macrophage cell lines (19), and in purified monocytes (31). We have observed gamma interferon-enhanced LPS activity in human whole blood (data not shown). Since many human pathological disease states involve the production of gamma interferon, it is reasonable to assume that this testing methodology and demonstration that compounds are not agonistic may have important implications in the development of novel therapeutics to treat these conditions.

Weak agonistic activity has been previously described for bacterially derived RSLA (21). Prior to the synthesis and testing of the proposed structure of the natural material, it was possible to ascribe weak agonistic activity to a "mixed antagonism" which has been reported for other lipid A analogs (2). Compounds exhibiting this property show antagonistic activity at lower concentrations, while at high concentrations or when added to sensitized (i.e., gamma interferon-treated) cells, they become agonistic. Lack of agonistic activity of the synthetic proposed structure (2′ *cis* LA), as well as the fact that bacterially derived RSLA is not structurally identical to any of the synthetic materials, makes it difficult to determine the site(s) on the bacterially derived RSLA molecule that is responsible for its agonistic activity. It is possible that agonistic activity is the result of some crucial unknown structural difference(s) between the bacterially derived RSLA and its proposed structure. Alternatively, it is possible that a small amount of an unknown potently agonistic substance is present as a contaminant in preparations of bacterially derived material. Analysis of possible contaminants in the bacterially derived RSLA is confusing, as evidenced by HPLC analysis (23). This material contains variants, including the saturated, unsaturated, and microheterogeneous forms, which may vary in chain length and position of the olefin (23). To date, saturated RSLA has been shown to lack agonistic activity (26), and our in-house structure-activity relationship studies of a similar class of compounds have confirmed this observation (unpublished results). The instability of bacterially derived RSLA or a contaminant

may give rise to agonistic components unique to the natural product. Recent results from our analysis of synthetically derived materials likely to be degradation products of 2′ *cis* LA indicate that agonistic components could arise by degradation of bacterially derived RSLA (unpublished results). As part of a previously described study (1), we found that while the synthetic materials were stable under conditions used to generate the sodium salt, rapid decomposition of bacterially derived RSLA occurred under these same conditions.

Bacterially derived RSLA has been shown to block a variety of LPS-mediated cellular events as well as initiate a broad range of biological effects which may be ascribed to agonistic activity (i.e., induction of tolerance to LPS and induction of glucocorticoids). In light of the discrepancies between agonistic and antagonistic activities of bacterially derived material and the synthetic analogs, we believe that further analysis of the activities of lipid A-like molecules may be warranted. The development of potent synthetic antagonists that demonstrate no in vitro or in vivo agonistic activity (13, 16, 28) may be helpful in further determining the biochemistry of endotoxin activity.

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