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Synthetic Lipid A with Endotoxic and Related Biological Activities Comparable to Those of a Natural Lipid A from an *Escherichia coli* Re-Mutant

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A synthetic compound (506), β(1-6) D-glucosamine disaccharide 1,4′-bisphosphate, which is acylated at 2′-amino and 3′-hydroxyl groups with (*R*)-3-dodecanoyloxytetradecanoyl and (*R*)-3-tetradecanoyloxytetradecanoyl groups, respectively, and has (*R*)-3-hydroxytetradecanoyl groups at 2-amino and 3-hydroxyl groups, exhibited full endotoxic activities identical to or sometimes stronger than those of a reference lipid A from an *Escherichia coli* Re-mutant (strain F515). Endotoxic activities tested include pyrogenicity and leukopenia-inducing activity in rabbits, body weight-decreasing toxicity in normal mice, lethal toxicity in galactosamine-sensitized mice and chicken embryos, and the preparation and provocation of the local Shwartzman reaction in rabbits. Compound 406, a synthetic counterpart of a biosynthetic precursor of lipid A molecule, showed by contrast only weak activities in all of the above assay systems except for the lethality in galactosamine-loaded mice. This finding strongly suggests that the presence of acyloxyacyl groups at the C-2′ and C-3′ positions of the disaccharide backbone is one of the most important determinant structures of the lipid A molecule for exhibition of strong biological activities characteristic of lipopolysaccharide and its lipid A moiety. The activities of the corresponding 4′-monophosphate (compound 504) and 1-monophosphate (505) analogs were considerably less than those of the parent molecule 506 and the reference F515 lipid A. Regarding other biological activities, not only compound 506 but also compounds 504, 505, and 406 showed definite activities, sometimes comparable to those of F515 lipid A and other reference natural products. These are the activation of *Tachypleus tridentatus* amoebocyte clotting enzyme cascade and human complement via the classical pathway, mitogenic and polyclonal B-cell activation of murine splenocytes, stimulation of peritoneal macrophages in a guinea pig, enhancement of migration of human blood polymorphonuclear leukocytes, and induction of a serum factor that is cytostatic and cytocidal to L-929 cells in *Mycobacterium bovis* BCG-primed mice. Relative potencies of test synthetic compounds depended on the assay systems and varied from one system to another. Dephospho-compound 503 lacked most of the biological activities that were definitely observed with phosphorylated compounds, probably because of its insolubility. This study demonstrates the successful chemical synthesis of an *E. coli*-type lipid A.

In the preceding papers (15, 24) of this series, we described the biological activities of lipid A analogs that were prepared as synthetic counterparts of the acidic, biosynthetic lipid A precursor (17, 18). One of them, compound 406 (Fig. 1), 2,2′-*N*-3,3′-*O*-tetraquis[(*R*)-3-hydroxytetradecanoyl]-β(1-6)-D-glucosamine disaccharide 1,4′-bisphosphate, was shown to exhibit distinct activities in all in vitro assays tested: *Limulus* activity, mitogenic and polyclonal B-cell activation (PBA) of murine splenocytes, stimulation of phagocytosis in murine peritoneal macrophages, and activation of human serum complement via the classical pathway. These activities were very close to those of the reference lipid A derived from *Escherichia coli* F515 (O8:K27, Re-mutant) in both effective dose and degree of stimulation. However, compound 406, like the biosynthetic lipid A precursor, was far less active in characteristic endotoxic properties such as pyrogenicity and the activity to prepare the local Shwartzman reaction, though the compound had as high a lethal toxicity as F515 lipid A for mice loaded with galactosamine (5). Galanos et al. (6) have reported essentially the same results in experiments with the same synthetic preparations.

The marked differences noted between the synthetic compound, 406, and the natural product, F515 lipid A, regarding biological activities that more or less characterize endotoxic lipopolysaccharide (LPS) and its lipid A moiety have been assumed to be attributable to the following fact: all four acyl groups bound to the amino groups at C-2 and C-2′ positions

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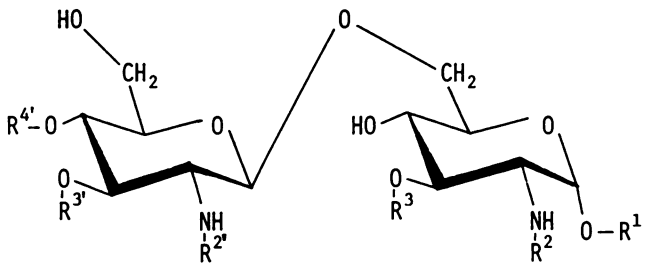

Compound	R^2	$R^{2'}$	R^3	$R^{3'}$	$R^{4'}$	R^1
503	C_{14}-OH	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-O-(C_{14})	H	H
504	C_{14}-OH	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-O-(C_{14})	P	H
505	C_{14}-OH	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-O-(C_{14})	H	P
506	C_{14}-OH	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-O-(C_{14})	P	P
406	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	P	P

FIG. 1. Chemical structure of test synthetic lipid A and its analogs. Abbreviations: P, $PO(OH)_2$; C_{14}-OH, (*R*)-3-hydroxytetradecanoyl; C_{14}-O-(C_{12}), (*R*)-3-dodecanoyloxytetradecanoyl; C_{14}-O-(C_{14}), (*R*)-3-tetradecanoyloxytetradecanoyl.

and the hydroxyl groups at C-3 and C-3′ positions of the disaccharide backbone are (*R*)-3-hydroxytetradecanoyl in compound 406 and its analogs (403 through 405), while the acyl groups at C-2′ and C-3′ positions of the backbone of *E. coli* and *Salmonella minnesota* lipid A's are (*R*)-3-acyloxyacyl (double acyl) types (9, 22).

The purpose of the present paper is to report that further synthetic study has accomplished the task of synthetic lipid A having full endotoxic and related biological properties virtually identical with those of the natural lipid A derived from Re-mutant *E. coli* F515.

MATERIALS AND METHODS

Animals. ICR, BALB/c, C57BL/6, and C3H/HeN mice were purchased from Charles River Japan, Osaka, Japan. BALB/c *nu/nu* and C3H/HeJ mice were obtained from Clea Japan, Osaka, Japan, and Jackson Laboratory, Bar Harbor, Maine, respectively. Domestic Japanese white rabbits and Hartley albino guinea pigs were supplied by Nihon Rabbit Co., Osaka, Japan.

Test materials. (i) Synthetic *E. coli*-type lipid A and its analogs. Compounds 504 through 506 (free acid or triethylamine [TEA] salt) and the dephospho-derivative compound 503 were synthesized as described in a separate paper (10) (Fig. 1). Compound 406, a synthetic counterpart of a biosynthetic lipid A precursor from *Salmonella* sp. (17) whose immunopharmacological activities were examined in the preceding studies (15, 24), was prepared as previously described (11) and served as one of the reference compounds.

(ii) Reference natural products. Specimens of reference standard lipid A (TEA salt) and Re-LPS (free acid) were prepared from *E. coli* F515 (O8:K27, Re-mutant) and generously supplied by O. Lüderitz and C. Galanos, Max-Planck-Institut für Immunobiologie at Freiburg, Federal Republic of Germany. Another reference product was a commercial LPS of *E. coli* O55:B5 prepared by a modification of the Westphal method and further purified by chromatography (lot no. 5, product no. 203; List Biological Laboratories, Inc., Campbell, Calif.). This preparation was chosen for its negligible mitogenicity to splenocytes of LPS-nonresponding C3H/HeJ mice, which distinguishes it from several other commercially available preparations of *E. coli* LPS.

Both synthetic and natural products were dissolved or suspended as homogeneously as possible at 2 mg/ml in 0.1% (vol/vol) TEA aqueous solution. Compounds 506 and 406, as well as the three natural products, were apparently clearly dissolved, compounds 504 and 505 were less soluble and gave a slightly turbid solution, and the dephospho-compound 503 was hardly dissolved but gave a fairly stable suspension. The stock solutions (or a suspension), kept at 4°C, were appropriately diluted with distilled water, physiological saline (saline), or cell culture medium before use for assays except in the following tests. In assays on pyrogenicity and leukopenia-inducing activity in rabbits, toxicity in normal mice, and preparatory and provocative activities for local Shwartzman reaction in rabbits, the above stock solution was diluted with an equal volume of aqueous solution (2 mg/ml) of bovine serum albumin (A-7511; Sigma Chemical Co., St. Louis, Mo.) and then appropriately diluted with saline. The bovine serum albumin solution did not provoke any febrile responses in rabbits at a dose of 31.6 μg/kg.

Lethal toxicity on galactosamine-loaded mice. The method of Galanos et al. (5) was followed for tests with galactosamine-loaded mice. In brief, groups of C57BL/6 mice (male, 8 to 10 weeks old) were sensitized by intraperitoneal injection of 16 mg of D-galactosamine hydrochloride (Wako Pure Chemicals, Osaka, Japan) in 0.5 ml of phosphate-buffered saline, followed immediately by intravenous injection in graded doses of test materials in 0.2 ml of distilled water or water alone. The death of mice due to intoxication was observed over a 24-h period.

Body weight-decreasing toxicity in normal mice. Groups of five ICR mice (male, 4 weeks old) were injected intraperitoneally with graded doses of test materials in 0.5 ml of the saline. The mice were allowed to take food and water ad libitum and were weighed before and daily after the injection

for 4 days by using an electric reading balance (Libro EB-2800; Shimadzu Co., Kyoto, Japan) to estimate the body weight changes caused by intoxication (12).

Lethal test on chicken embryos. This was performed by the method of Finkelstein (4). Groups of chicken embryos (11 days old, white Leghorn, ISA Babcock-B300; Maruyama Poultry Farm, Kagawa, Japan) were injected intravenously with 0.1 ml of test solutions appropriately diluted with distilled water and observed for 48 h, and 50% lethal doses were calculated by the Kärber method (13).

Pyrogenicity. Pyrogenicity was tested as described in Japanese Pharmacopeia, using Japanese domestic white rabbits (weight, 2 to 2.5 kg) (24).

Leukopenia-inducing activity. Blood specimens were drawn from domestic Japanese white rabbits (weight, 2 to 2.5 kg) at appropriate intervals from the marginal ear vein with a heparinized disposable syringe before and after intravenous injection of test materials. Blood specimens (20 μl) were diluted with 10 ml of the diluent for the blood counter (model CEO310; TOA Medical Electronics Co., Tokyo, Japan), and then 3 drops of Quick Lyser (QL-21; TOA) were added to lyse the erythrocytes. Leukocyte number (cells per cubic millimeter) was determined with a Microcell counter (model CC-130; TOA).

Preparative and provocative activities for Shwartzman reaction. Testing for preparative and provocative activities was carried out with Japanese white rabbits (female, weighing around 3 kg) by the conventional methods (24).

Assay for immunoadjuvant activity. (i) Mice. The methods described by Šterzi and Říha (23) and Dresser and Wortis (3) for assay of immunoadjuvant activity were essentially followed. Groups of 5 BALB/c mice (male, 8 weeks old) were immunized by intraperitoneal injection of 2×10^7 sheep erythrocytes (SRBC) in 1.0 ml of phosphate-buffered saline added with 50 μg of test materials (none in control). One week later, spleens were removed from the immunized mice and minced on a cytosieve. Splenocytes thus obtained were suspended in RPMI 1640 medium (Research Foundation for Microbial Diseases, Osaka University, Osaka, Japan). Portions (100 μl, 10^6 cells) of the above cell suspension were distributed in 96-well microculture plates (Corning Glass Works, Corning, N.Y.) and then added with 10 μl of packed SRBC and 10 μl of fresh guinea pig serum adsorbed with SRBC as complement (and further supplemented with 10 μl of a rabbit anti-mouse immunoglobulin G serum [1:30; Cappel Laboratories, Cochranville, Pa.] in an indirect assay). The reaction mixture was put in a Cunningham chamber and incubated under a mixture of CO_2 and air at 37°C for 1 h. The number of hemolytic plaque-forming cells (PFC) was counted with the naked eye. Immunoadjuvant activity of test materials was expressed as the stimulation index, the ratio of the number of direct (or indirect) PFC per spleen in test group to that in the control group.

(ii) Guinea pigs. Five Hartley albino guinea pigs (female; weight, about 300 g) per group were immunized by intrafootpad injection of test materials (100 μg per animal) and crystalline ovalbumin (1 mg; grade V; Sigma) emulsified with Freund incomplete adjuvant (Difco Laboratories, Detroit, Mich.). The induction of delayed-type hypersensitivity (DTH) was examined by a corneal test 3 weeks after immunization, and anti-ovalbumin precipitin antibody level in serum was estimated by the quantitative precipitin method 4 weeks after the immunization.

Induction of a serum factor that is cytostatic and cytocidal to L-929 cells in BCG-primed mice. Groups of 3 ICR mice (female, 5 weeks old) were primed by intravenous inoculation of 1.3×10^8 viable units (in 0.2 ml of saline) of freeze-dried, percutaneous *Mycobacterium bovis* BCG vaccine (Japan BCG Laboratory, Tokyo, Japan) (2, 8). Each group received an intravenous injection of 10 or 2 μg (in 0.2 ml of saline) of test compounds 2 weeks after the priming. After 90 min, blood samples were drawn, and separated sera were inactivated by heating at 56°C for 30 min and pooled for each group. If it was necessary, pooled serum specimens were kept at −20°C until use. Cytostatic and cytocidal actions of test sera on L-929 cells (NCTC clone 929; kindly supplied by Research Laboratories, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) were determined as follows. Each well of a 96-well microculture plate (Corning) was filled with 90 μl (containing 3×10^4 cells) of the suspension of L-cells, grown and suspended in Eagle minimum essential medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml; Research Foundation for Microbial Diseases, Osaka University, Osaka, Japan), 10% fetal bovine serum (Flow Laboratories Inc., McLean, Va.), and 2% meylon (Ohtsuka Seiyaku Co., Tokyo, Japan). After 2 h, 100 μl of serially fivefold-diluted serum specimens to be tested were added to each well, and then [^{3}H]thymidine (0.5 μCi/10 μl; Biochemical Center, Amersham, Buckinghamshire, England) was added. After cultivation for 48 h at 37°C under 5% CO_2 in air and subsequent centrifugation, the supernatant was carefully removed, and 1 drop of 0.2% trypsin–0.02% EDTA (aqueous solution) was added. After 3 min, L-cells detached from the well were harvested with a semiautomatic multiple-cell harvester (LM-101, LABO MASH; Labo science Co., Tokyo, Japan). The culture was done in quadruplicate. Thymidine uptake into L-929 cells was measured by the conventional scintillation method. The cytostatic titer, or 50% effective dose, refers to a reciprocal of the final dilution of test serum that results in 50% inhibition of thymidine uptake of L-cells. This value was calculated by the Kärber method (13). For the cytocidal test, L-929 cells (5×10^3) were cultured with a 1:100 dilution of test sera in 1 ml of Eagle minimum essential medium supplemented as described above for 48 h at 37°C in a 24-well microculture plate (Corning). The culture plates were centrifuged at $100 \times g$ for 10 min to collect detached cells. To each well containing adherent and sedimented L-929 cells was added 0.1 ml of a mixture of 0.4% trypsin–0.04% EDTA and 0.25% trypan blue (1:1 [vol/vol]). Cell suspensions thus obtained were put in a hemacytometer and examined for the number and viability of cells by microscopy. Cytocidal activity was graded by criteria described in footnotes *b* and *c* of Table 8.

***Limulus* test.** The *Limulus* test was performed with Pre Gel, an amoebocyte lysate of *Tachypleus tridentatus* (Seikagaku Kogyo Ltd., Tokyo, Japan) and with Toxicolor test (Seikagaku Kogyo Ltd.). Details of the latter test have been described elsewhere (21, 24). Briefly, a mixture of a lyophilized lysate of *T. tridentatus* and a chromogenic substance, *N-tert*-butoxycarbonyl-L-leucyl-glycyl-L-arginine-*p*-nitroaniline, was used as a reagent. An LPS from *E. coli* O111:B4 (LPS-W; Difco) was used as a reference standard in both tests.

Activation of human complement. A pooled, fresh, adult human serum, kept at −70°C, was incubated with an equal volume of test material solution or suspension appropriately diluted with saline. After incubation at 37°C for 1 h, the decrease in total hemolytic activity of the complement by test compounds was determined by one-point method that was based on the original hemolysis method of Mayer but simplified by a single colorimetric determination (Kitamura et al. [14]). The blocking effect on the classical pathway by

the addition of 10 mM EGTA [ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] plus 5 mM $MgCl_2$ was also determined.

Stimulation of macrophages. (i) Collection of peritoneal macrophages. A Hartley albino guinea pig (female, weighing around 500 g) was intraperitoneally injected with sterile liquid paraffin (Wako Pure Chemicals). After 4 to 7 days, the peritoneal cavity of the animal was washed twice with Hanks solution (Nissui Pharmaceutical Co., Tokyo, Japan), and the cell suspension thus obtained was washed three times with Hanks solution by centrifugation. Portions (0.5 ml) of the peritoneal exudate cells suspended in RPMI 1640 medium (Research Foundation for Microbial Diseases, Osaka University) supplemented with 10% fetal bovine serum (10^6 cells per ml) were distributed in each well of a 24-well microculture plate (Falcon 3047; Becton Dickinson and Co., Paramus, N.J.) incubated under 5% CO_2 in air at 37°C for 2 h, and each well was washed twice with Hanks solution to remove nonadherent cells. The cells attached to the culture plate served as oil-induced peritoneal macrophages (20).

(ii) Assay for thymidine and glucosamine incorporation. Each well with attached macrophages was added with appropriate dilution (10 μl) of test materials (none in control), and the culture plate was incubated under 5% CO_2 in air at 37°C for 48 h. During the final 24 and 6 h of the culture, the macrophages were pulsed with 0.2 μCi of [*methyl*-^{3}H]thymidine and 0.1 μCi of D-[U-^{14}C]glucosamine hydrochloride (New England Nuclear Corp., Boston, Mass.), respectively. After completion of the culture, 10% trichloroacetic acid solution (0.5 ml) was added to stop the reaction, and the macrophages were detached with a rubber policeman and washed with 10% trichloroacetic acid. The trichloroacetic acid-insoluble fraction was solubilized with Protosol (New England Nuclear Corp.) at room temperature overnight. Incorporation of [^{3}H]thymidine and [^{14}C]glucosamine was measured by double count with a liquid scintillation spectrometer (LSC-673, Aloka Co., Tokyo, Japan) by the conventional scintillation method. Duplicate or triplicate cultures were made for each experimental point to obtain the mean ± standard error (SE) of the percent inhibition for thymidine uptake [1 − (disintegrations per minute in a test culture/disintegrations per minute in the control culture)] × 100 and the stimulation index for glucosamine uptake (a ratio of disintegrations per minute in a test culture to disintegrations per minute in the control) (20).

(iii) Macrophage spreading. Macrophage spreading was determined by the method described by Tanaka et al. (25). In brief, the total and spreading macrophages in each well, which were cultured overnight as described above, were counted under an inverted phase-contrast microscope. The extent of spreading is given by the following equation: percent spreading = (number of spreading cells/number of total attached cells) × 100. Macrophages were considered spread when their membrane apron occupied an area double that of unspread cells or when they were elongated.

(iv) Enhancement of superoxide anion (O_2^-) generation. Test compounds were examined for the enhancing effect on the release of O_2^- by oil-induced macrophages in response to wheat germ agglutinin and cytochalasin E by the method of Yagawa et al. (26) with minor modifications. Briefly, macrophage monolayers (5×10^5 cells per well) on a 24-well microculture plate were incubated with indicated doses of synthetic and natural compounds in 0.5 ml of RPMI 1640 medium for 48 h as described, in duplicate. The macrophages exposed to test materials were washed with Hanks solution and preincubated with 1 ml of the assay mixture containing 50 μM of cytochrome *c* (from horse heart, Type VI; Sigma) in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer at 37°C for 10 min. Ten microliters each of wheat germ agglutinin (4 mg/ml; Sigma) and cytochalasin E (500 μg/ml; Sigma) were then added, and the culture plate was incubated at 37°C for 15 min. The incubation was done by using Micro Mixer (MX-4; Sanko Junyaku Co., Tokyo, Japan) to accelerate contact of macrophages with the reagents. All the above incubation was done under 5% CO_2 in air. The rate of reduction of cytochrome *c* in the reaction mixture was measured by recording the absorption at 550 to 540 nm with a double-beam spectrophotometer (model 200-20; Hitachi Ltd., Tokyo) and expressed as nanomoles per minute per 10^6 cells (molar absorption coefficient, 19.1×10^3).

TABLE 1. Lethal toxicity of synthetic lipid A, its analogs, and reference natural products on galactosamine-loaded mice

Compound[a]	No. of deaths/no. of mice tested with the following dose (ng):					LD_{50} (ng)[b]
	1,000	100	10	1	0.1	
503	0/5					>1,000.0
504		5/5	1/5	0/5		20.0
505	5/5	4/5	0/5			50.1
506		5/5	3/5	0/5		7.9
406		5/5	7/15	0/5		10.8
Lipid A		5/5	1/5	0/5		20.0
Re-LPS			5/5	2/5	0/5	1.3
LPS		5/5	5/5	0/5		3.2

[a] Lipid A and Re-LPS were prepared from *E. coli* O8:K27, Re-mutant (strain F515), and LPS is a commercial product from *E. coli* O55:B5 (List). The same preparations were used as reference natural products in the assays presented in the following tables and figures unless otherwise noted.

[b] LD_{50}, 50% Lethal dose, calculated by the Kärber method.

Stimulation of murine splenocytes. (i) Stimulation of thymidine uptake. Splenocytes of BALB/c *nu/nu* (male, 8 weeks old), C3H/HeN (male, 9 weeks old), and C3H/HeJ (male, 9 weeks old) mice were incubated with graded doses of test materials in RPMI 1640 medium (without fetal bovine serum) at 37°C for 24 h and then, after the addition of [^{3}H]thymidine (New England Nuclear Corp.), were further cultivated at 37°C for 24 h under 5% CO_2 in air (24). The stimulatory effect was expressed as a ratio of disintegrations per minute in a test culture to disintegrations per minute in the respective control culture.

(ii) Polyclonal B-cell activation. This was determined as described in a previous paper (16), except for that 2,4,6-trinitrophenylated (TNP) SRBC were used instead of SRBC as a target. Briefly, splenocytes (6×10^6 cells) of BALB/c mice (male, 8 weeks old) were incubated with appropriate doses of test materials in RPMI 1640 medium supplemented with fetal bovine serum. After the incubation under 5% CO_2 in air at 37°C for 3 days, the number of hemolytic PFC against TNP-SRBC was determined in a Cunningham chamber. PBA ability of test materials was expressed as the ratio of PFC count in a test culture to that in the control, i.e., as a stimulation index. All determinations were carried out in hexaplicate cultures.

Enhancement of migration of human PMNL. Details of the procedure for measuring enhancement of migration of human polymorphonuclear leukocytes (PMNL) were described in a previous paper (16). Briefly, this assay was performed with a multiwell chemotaxis assembly (Neuro Probe, Cabin John, Md.) with human PMNL enriched from heparinized peripheral blood. Determination was made of the number of

TABLE 2. Lethal toxicity of synthetic lipid A, its analogs, and reference natural products on 11-day-old chicken embryos

Compound	No. of deaths/no. of embryos tested with the following dose (ng):						LD_{50} (ng)[a]
	20,000	10,000	2,000	200	20	2	
503	0/11						>20,000
504		2/10	2/11				—[b]
505		0/9	0/10				>10,000
506			13/14	9/9	0/9		74
406		0/10	0/12				>10,000
Lipid A				8/11	5/10	0/10	~37
Re-LPS				11/11	6/9	0/8	14
LPS			9/9	5/11	3/10	0/11	111

[a] 50% lethal dose, calculated by the Kärber method.
[b] —, Not calculable.

PMNL which migrated to the surface of the membrane sheet between the upper and lower well, adjacent to the lower well with an added test specimen in triplicate for each experimental point. The migration enhancing effect was expressed as a ratio of PMNL number in a test to that in the respective control. Fresh human serum samples (diluted, 1:10) activated by incubation with LPS-B of *Salmonella enteritidis* (Difco) and 10^{-6} M *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (Sigma) were used as positive controls.

Other procedures. Meticulous precautions were exercised to avoid extraneous contamination of experimental ware and reagents with LPS. Statistical significance of the difference between each test and the respective control was examined by the Student's *t* test in most of the in vitro assays.

RESULTS

Endotoxic activities. (i) Lethal toxicity. Assays were made with the mice which were rendered highly susceptible to LPS by galactosamine loading (5) and with chicken embryos that were naturally highly sensitive to LPS, because availability of test synthetic compounds was very limited. Compounds 506 and 406 were markedly toxic in galactosamine-loaded mice (Table 1). Their lethality was slightly stronger than that of F515 lipid A but moderately weaker than those of F515 Re-LPS and *E. coli* O55:B5 LPS. Monophosphorylated analogs of compound 506 (4'-monophosphate, 504; 1-monophosphate, 505) were considerably less toxic than compound 506. Compound 503, a dephospho-analog, was

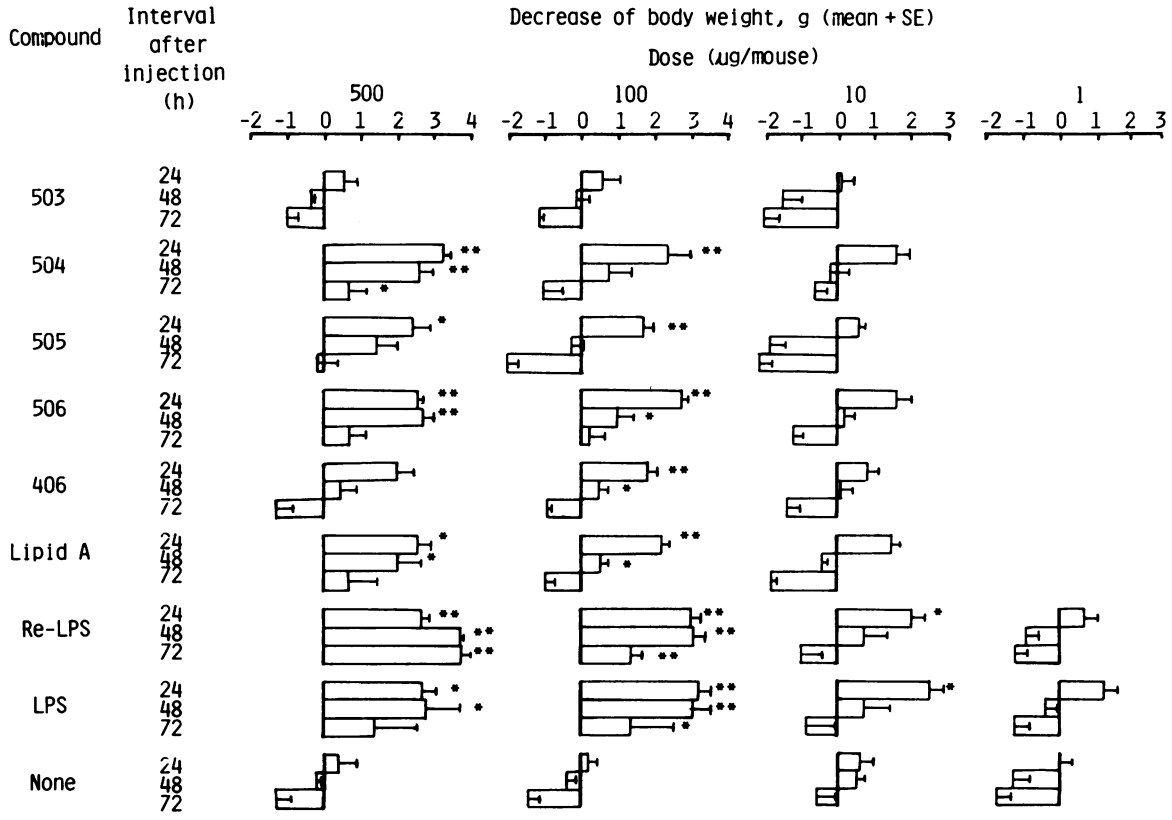

FIG. 2. Effects on body weight of synthetic lipid A, its analogs, and reference natural products. See the text for experimental conditions. One mouse of five receiving 500 μg of compound 506 died between 24 and 48 h. Significant difference from control value: *, $P < 0.05$; **, $P < 0.01$.

TABLE 3. Pyrogenicity and leukopenia-inducing activity of synthetic lipid A, its analogs, and reference natural products

Compound, dose (μg/kg)	Febrile response			Leukopenia (no. positive/no. tested)[a]
	No. positive/no. tested[b]	Change in temp (°C)[c]		
		Peak I	Peak II	
503				
31.6	0/3	−0.18	0.30	ND[d]
10.0	0/3	−0.22	0.30	ND
1.0	0/3	−0.08	0.10	ND
504				
1.0	2/2	1.46	1.03	2/2
0.1	1/3[e]	0.22	0.41	0/3
0.01	0/1	0.20	0.43	0/1
505				
1.0	1/3[e]	0.51	0.48	0/3
0.1	1/2	0.24	0.46	0/2
0.01	0/1	0.08	0.16	0/1
506				
1.0	4/4	0.87	1.20	2/2[f]
0.1	3/4	0.79	0.86	2/3[f]
0.01	2/2	0.78	1.23	2/2
0.001	0/2	0.33	0.48	0/2
406				
10.0	1/1	0.65	0.95	ND
1.0	3/4	0.75	0.53	1/3[f]
0.1	0/3	0.20	0.21	0/3
Lipid A				
1.0	3/3	1.03	1.08	1/1[f]
0.1	1/3	0.23	0.38	3/3
0.01	0/2	0.29	0.36	0/2
Re-LPS				
0.1	1/3[g]	1.06	1.78	3/3
0.01	1/2	0.40	0.73	1/2
0.001	0/3	−0.03	−0.16	0/3
LPS				
0.1	1/2	0.75	0.64	2/2
0.01	1/2	0.66	0.38	1/2
0.001	0/2	0.30	0.10	0/2

[a] A positive result was defined as a decrease of circulating blood leukocytes over 33% of the value before injection of test materials.
[b] A positive result was defined as an increase in rectal temperature of more than 0.6°C.
[c] The mean temperature increase in all test rabbits, irrespective of positive and negative febrile response, with some exceptions (see footnotes *e* and *g*). Peak I, 1 to 1.5 h; peak II, 2.5 to 4 h.
[d] ND, Not done.
[e] One of three rabbits showed significant temperature decrease of ca. 1°C. This rabbit was excluded in calculation of the mean temperature changes.
[f] Some of the rabbits examined for febrile response were not submitted to circulating blood leukocyte count.
[g] Two of three rabbits showed a marked temperature decrease of ca. 2°C. These rabbits were not included in calculation of the mean temperature changes.

not lethal. In the assay with chicken embryos, however, marked differences in lethality between compounds 506 and 406 were noted (Table 2). The 50% lethal dose of compound 506 was 74 ng, close to that of F515 lipid A (ca. 37 ng), while compound 406, like compounds 505 and 503, did not show any lethality up to the maximum test dose of 10 μg. Compound 504 exerted a weak lethality. F515 Re-LPS and O55:B5 LPS were considerably more toxic and slightly less toxic than compound 506, respectively.

TABLE 4. Activity of synthetic lipid A, its analogs, and reference natural products to prepare rabbit skin for local Shwartzman reaction[a]

Compound	No. of hemorrhagic sites/no. of sites tested with the following dose (μg per site):				
	80	40	20	10	5
503	0/3	0/3			
504		2/3	0/3	0/3	
505	1/3	1/3	1/3		
506		4/5	2/3	1/3	1/3
406	0/3	0/3			
Lipid A			3/3	2/3	1/3
Re-LPS			3/3	2/3	2/3

[a] Skin sites of rabbits were prepared by intracutaneous injection of indicated doses of test compounds (in 0.2 ml of saline). Twenty hours later, the rabbits received an intravenous injection of 100 μg of *S. minnesota* R595 Re-LPS (in 1 ml of saline) per rabbit for provocation.

(ii) Effect on body weight in normal mice. Compounds 506 and 504, as well as the three reference natural products, caused a marked loss in body weight of more than 15% which lasted for 24 to 48 h at doses of 500 and 100 μg (Fig. 2). There was practically no difference between compound 506 and the reference F515 lipid A in body weight-decreasing action. Compound 504 was slightly less toxic than 506 when comparison was made at a dose of 100 μg. Compounds 505 and 406 were considerably less toxic than 506. Compound 503 completely lacked this toxicity. The body weight decreases induced by F515 Re-LPS and O55:B5 LPS were considerably higher than those induced by compound 506 and F515 lipid A. The minimum effective dose of the former two compounds was around 10 μg, whereas that of the latter two was 100 μg. Most of the mice receiving 500 and 100 μg of F515 Re-LPS and O55:B5 LPS and some of the mice receiving 500 μg of compound 506 were severely sick, with discharge from the eyes and intestinal disorders which lasted 1 to 2 days. However, all the sick animals recovered 3 days after the start of the assay, except for one in the 506 group which died between the 24- and 48-h readings.

(iii) Pyrogenicity and leukopenia-inducing activity. Compound 506 was found to be considerably more pyrogenic than the reference F515 lipid A and O55:B5 LPS (Table 3). The pyrogenicity of 506 was slightly stronger than that of F515 Re-LPS, and they caused distinct and detectable febrile responses, respectively, at a dose of 0.01 μg/kg. A dose of compound 406 nearly 100 times higher than that required for 506 was needed to produce a definite pyrogenic effect.

TABLE 5. Activity of compounds 506, 406, and *E. coli* F515 lipid A to provoke the local Shwartzman reaction in rabbits[a]

Preparatively injected compound (μg per site)	No. of hemorrhagic skin sites/no. of test sites after provocation by the following compound		
	506	406	F515 lipid A
506 (20)	2/3	0/2	2/3
Lipid A (20)	3/3	0/2	3/3
Re-LPS (10)[b]	3/3	0/2	3/3

[a] Groups of rabbits received a preparative, intracutaneous injection of compound 506, 406, pr Re-LPS. Twenty hours later, the rabbits were injected intravenously with 100 μg of test compound per rabbit.
[b] A specimen prepared from *S. minnesota* R595.

TABLE 6. Activation of the clotting enzyme cascade of *T. tridentatus* amoebocyte lysate by synthetic lipid A, its analogs, and reference natural products

Compound	Minimum effective dose (ng per test)[a]	Equivalent of reference LPS (ng/mg)[b]
503	100	1,130
504	0.01	830,000
505	0.1	44,000
506	0.01	2,380,000
406	0.01	1,340,000
Lipid A	0.01	1,480,000
Re-LPS	0.01	6,650,000
LPS	0.01	770,000

[a] Data shown were obtained by the Pre Gel test.
[b] Data shown were obtained by the Toxicolor test. The reference LPS was Lipopolysaccharide W of *E. coli* O111:B4 (Difco). One nanogram of this LPS is equivalent to 2.9 endotoxin units of USP reference standard endotoxin prepared from *E. coli* O113 in the Toxicolor test.

Compounds 504 and 505 were considerably less pyrogenic than 506, and compound 503 did not cause a detectable febrile response at a dose as high as 31.6 μg/kg.

The positive febrile responses caused by pyrogenic doses of test synthetic compounds were generally accompanied by a significant decrease in the number of circulating blood leukocytes (leukopenia) as noted in rabbits receiving the reference natural products, though leukopenic response seemed to be slightly less affected than febrile response.

(iv) Preparation and provocation of the local Shwartzman reaction. Intracutaneous injection of compound 506 prepared the rabbit skin to be provoked by subsequent intravenous administration of *S. minnesota* R595 Re-LPS (100 μg per rabbit; prepared in the laboratory of Kasai) (Table 4). The preparatory activity of compound 506 seemed to be slightlyweaker than those of the reference F515 lipid A and F515 Re-LPS. Neither 503 nor 406 was capable of preparing the Shwartzman reaction at the maximum dose examined (80 μg per site), while compounds 504 and 505 showed weak but detectable preparatory ability. Provocative activity of compounds 506 and 406 was compared with F515 lipid A (Table 5). Compound 506 clearly provoked a hemorrhagic reaction at the sites that had been prepared by intracutaneous injection of compound 506, F515 lipid A, and *S. minnesota* R595 Re-LPS, but compound 406 lacked such activity.

***Limulus* test.** The ability of synthetic compounds and reference natural products to activate the clotting enzyme cascade of *T. tridentatus* (a horseshoe crab) amoebocyte lysate was tested by the conventional Pre Gel test and the Toxicolor test (Table 6). Compounds 506, 504, and 406 exerted powerful activity comparable to those of the three natural products in the Pre Gel test. In the Toxicolor test, a recently developed colorimetric method in which a chromogenic substance (22) was used, the activity of 506 was shown to be stronger than that of 406, which had the same level of activity as F515 lipid A. However, the activity of 505 was about three times less than that of F515 Re-LPS. The activity of 504 was about one-third of that of 506. Compound 505 showed weak activity, and the dephospho-analog (503) was practically inactive. The activity of O55:B5 LPS was three-fourths of that of *E. coli* O111:B4 LPS which was used in both Pre Gel and Toxicolor tests as a positive standard.

Other biological activities. (i) Activation of human complement. Compounds 504 through 506 activated the complement cascade in human serum as effectively as did F515 lipid A and Re-LPS (Fig. 3). There are no substantial differences among the dose-response curves given by the above synthetic and natural compounds. Compound 406 and O55:B5 LPS were considerably less active in complement activation, and 503 was hardly active. We then attempted to assess whether the complement activation by compound 506, F515 lipid A, F515 Re-LPS, and O55:B5 LPS was influenced by blocking the classical pathway by addition of EGTA (10 mM) and $MgCl_2$ (5 mM) to the complement serum. Essentially no activation of complement occurred with any of test compounds (Fig. 4). This finding indicates that complement activation by compound 506, as well as that by reference natural products including *E. coli* O55:B5 LPS, proceeds almost exclusively via the classical pathway.

(ii) Stimulation of macrophages. Synthetic compounds 506 and 504 strongly stimulated adherent peritoneal exudate cells (macrophages) of a guinea pig to inhibit thymidine uptake, to increase glucosamine incorporation, and to enhance spreading on a culture plate, as did the three reference natural products (Fig. 5). Compounds 505 and 406 were

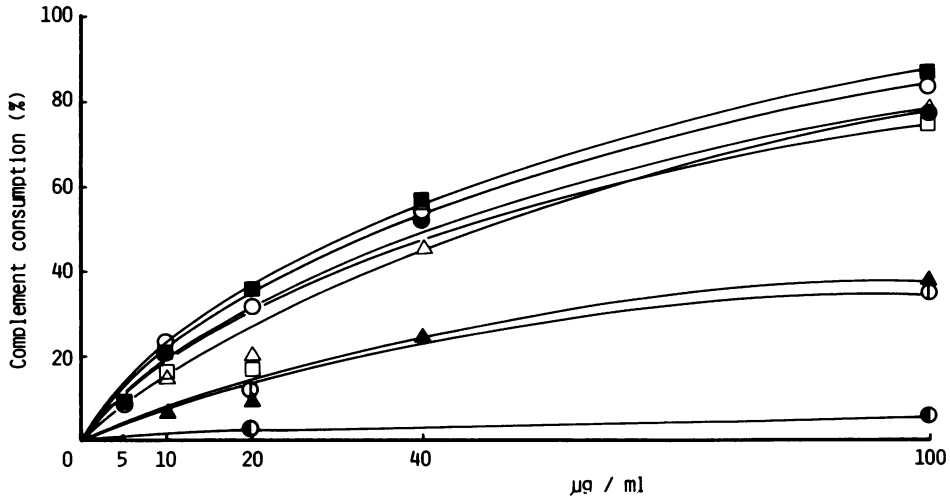

FIG. 3. Activation of human serum complement by synthetic lipid A, its analogs and reference natural products. Symbols: ◐, compound 503; □, 504; ■, 505; ○, 506; ⓓ, 406; ●, F515 lipid A; △, F515 Re-LPS; ▲, O55:B5 LPS.

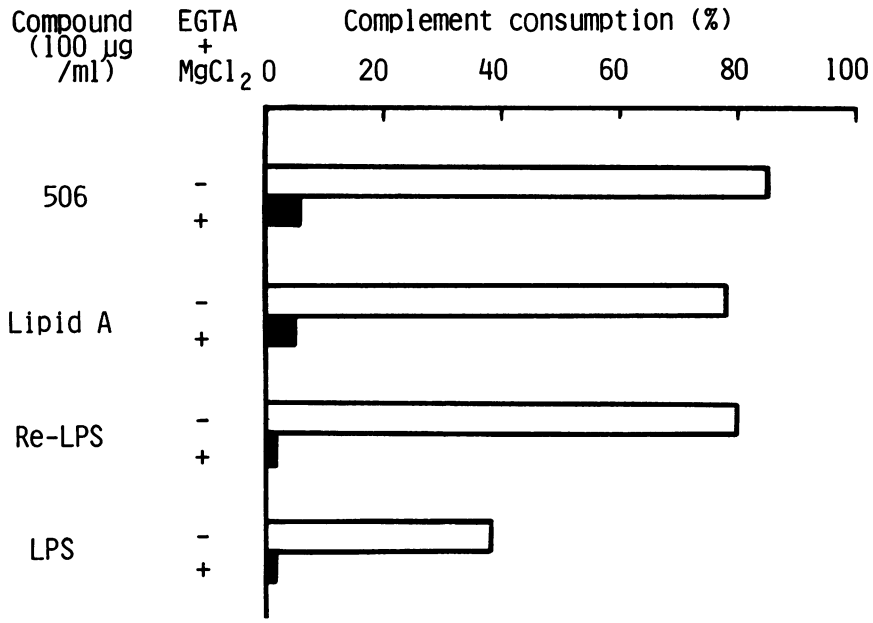

FIG. 4. Blocking effect of the classical pathway by addition of EGTA (10 mM) and $MgCl_2$ (5 mM) on complement activation by compound 506 and reference natural products (1 mg/ml).

stimulatory to the macrophages but considerably less active than 506. Compound 503 was inactive in this assay. Relative potency to stimulate macrophages among synthetic and natural compounds was confirmed by determination of dose (0.1 to 1,000 ng/10^6 cells)-response relationships. Compound 506 gave a pattern, in terms of the extent of stimulation in response to decreasing doses, comparable to that given by F515 lipid A. F515 Re-LPS and O55:B5 LPS were slightly more stimulatory to macrophages than were compound 506 and F515 lipid A, when the minimum effective doses were compared, i.e., 1 to 10 μg in the former two compounds and 10 μg in the latter two (data not shown).

Stimulatory effects of synthetic lipid A and its analogs on guinea pig peritoneal macrophages were also demonstrated in terms of the enhancement of superoxide anion generation by pretreatment of the cells. Synthetic judgement of extent of stimulation and effective dose indicates that among test synthetic compounds, 506 showed the strongest activity, comparable to that of F515 lipid A and slightly stronger than that of O55:B5 LPS, and that the activity of 504 was slightly higher than that of 505 (Table 7).

(iii) Mitogenic effects on murine splenocytes. The test synthetic compounds (except 503) significantly increased the uptake of thymidine by splenocytes of BALB/c *nu/nu* mice

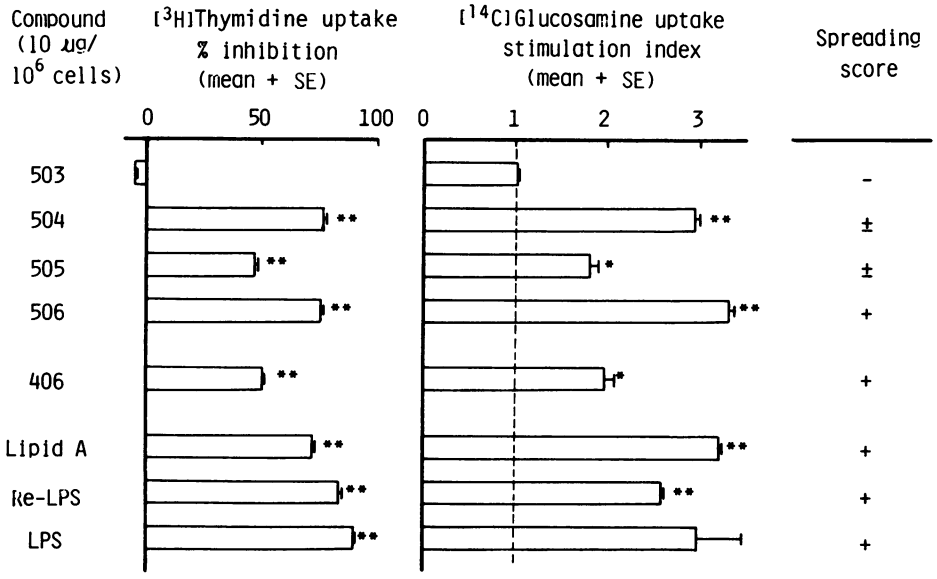

FIG. 5. Stimulatory effects of synthetic lipid A and its analogs on peritoneal exudate macrophages of a guinea pig compared with those of reference natural products. The counts of [^{3}H]thymidine and [^{14}C]glucosamine uptakes (mean ± SE) in control cultures were 65,452 ± 1,575 and 887 ± 24 dpm, respectively. Spreading was arbitrarily scored as follows: −, <10%; ±, 10 to <30%; +, ≥30%.

TABLE 7. Enhancing effects of the pretreatment of guinea pig peritoneal macrophages by synthetic lipid A, its analogs, and reference natural products on superoxide anion generation of the cells

Compound, dose (μg)	Enhancement effect (nmol/min per 10^6 cells)[a]	Stimulation index[b]
504		
10	131 ± 19	0.5
1	1,521 ± 80*	5.7
0.1	1,526 ± 156*	5.7
0.01	1,160 ± 45*	4.3
505		
10	994 ± 112*	3.7
1	1,274 ± 147*	4.7
0.1	714 ± 100*	2.7
0.01	154 ± 7	0.6
506		
10	583 ± 64*	1.9
1	1,477 ± 206*	5.5
0.1	1,587 ± 23*	5.9
0.01	1,302 ± 138*	4.8
Lipid A		
10	999 ± 90*	3.7
1	1,617 ± 48*	6.0
0.1	1,559 ± 120*	5.8
0.01	1,160 ± 66*	4.3
LPS		
10	1,036 ± 102*	3.9
1	1,232 ± 57*	4.6
0.1	1,097 ± 94*	4.1
0.01	905 ± 60*	3.4
None (control)	269 ± 52	1.0

[a] Expressed as mean ± standard error. Asterisks indicate values significantly different ($P < 0.01$) from the control.
[b] Ratio of O_2^- generation in a test culture to that in the control.

(Fig. 6), indicating that they were powerful B-cell mitogens, as were the reference natural products. Among synthetic compounds, 406 exerted the highest activity, which was close to that of F515 lipid A but considerably less than the activities of other two natural products. The mitogenicity of compound 505 was comparable to that of 506 and stronger than that of 504. Dephospho-compound (503) was inactive as in most of the other assays. The test synthetic and natural compounds similarly stimulated the thymidine uptake of splenocytes of C3H/HeN mice, but to a lesser extent than in those of BALB/c *nu/nu* mice. In sharp contrast, none of them significantly affected splenocytes of LPS-nonresponding C3H/HeJ mice, although compound 505 and O55:B5 LPS showed marginally detectable stimulation (data not shown).

(iv) PBA activity. All synthetic compounds, including the dephospho-compound (503), markedly increased anti-TNP-SRBC hemolytic PFC counts in the spleen of BALB/c mice (Fig. 7). The activities of compounds 504 through 506 seemed to be stronger than those of compound 406 and F515 lipid A at doses of 1 and 10 μg/6 × 10^6 splenocytes. At a dosage level of 0.1 μg, compound 506 showed slightly stronger PBA activity than F515 lipid A or O55:B5 LPS. F515 Re-LPS was the most stimulating among test compounds at this dose level.

(v) Enhancement of PMNL migration. All test synthetic compounds enhanced the migration of PMNL derived from human peripheral blood towards themselves, as did the reference natural products (Fig. 8). When effective dose and extent of stimulation were considered together, the activity of compound 506 was comparable to those of F515 lipid A and Re-LPS, and stronger than those of compound 406 and O55:B5 LPS.

(vi) Immunoadjuvant activities. Synthetic lipid A and its analogs, except 503 (at a dose of 50 μg), markedly increased specific anti-SRBC hemolytic PFC counts, either direct or indirect, in the spleens of BALB/c mice that had been immunized by intraperitoneal injection of SRBC and test compounds (Fig. 9). There were no significant differences in the extent of stimulation effected by compounds 504 through 506 and compound 406. However, the adjuvant activities of these compounds were weaker than those of F515 lipid A and O55:B5 LPS. A seemingly low potency of F515 Re-LPSwas partly due to its high toxicity in the mice, as indicated by the finding that two of five mice died within a few days after the immunization. Intrafootpad injections of compounds 506 and 504 (100 μg) with ovalbumin (1 mg per animal) in Freund incomplete adjuvant (Difco) induced distinct DTH to ovalbumin in terms of a definitely positive corneal reaction and a markedly elevated antiovalbumin precipitin antibody level in serum (Fig. 10). The adjuvant activities of compounds 506 and 504 to increase antibody production were stronger than those of F515 Re-LPS and

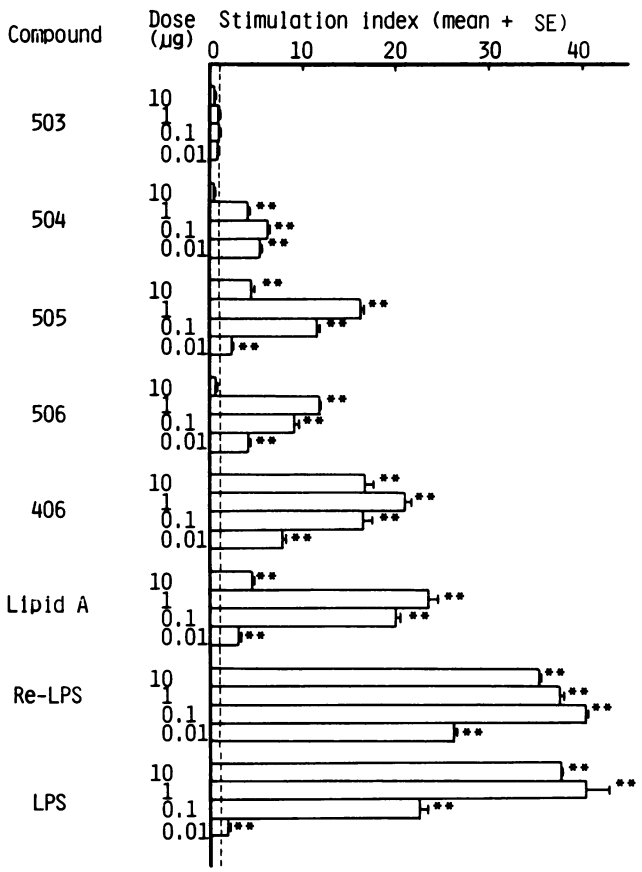

FIG. 6. Stimulation of thymidine uptake of BALB/c *nu/nu* mouse splenocytes by synthetic lipid A, its analogs, and reference natural products. Doses are shown in micrograms per 5 × 10^5 cells. The counts (mean ± SE) in control cultures were 1,434 ± 34 dpm.

O55:B5 LPS, while a reverse trend was observed in DTH-inducing activity. In the present assay, F515 lipid A showed some but not significant activity in both induction of DTH and elevation of antibody level. Compounds 503 and 505 were practically inactive. Compound 406, whose adjuvant activity could not be examined along with the newly synthesized compounds because of its limited availability, showed distinct adjuvant activities comparable to that of 506 in both DTH induction and antibody production (average corneal score, 1.7; serum antibody level [ratio to control], 4.3) in a similar assay described previously (24).

(vii) Induction of a serum factor cytostatic and cytocidal to L-929 cells in BCG-primed mice. Test compounds, except 503, exhibited the distinct activity to induce a serum factor that exerted cytostatic and cytocidal actions on cultured L-929 cells (a possible tumor necrosis factor [2, 8]) by intravenous injection in ICR mice which had been primed by BCG inoculation 2 weeks in advance (Table 8). When the degree of cytostatic and cytocidal actions and effective dose were considered together, the tumor necrosis factor-inducing activity of compound 506 was slightly lower than those of the three natural products but somewhat higher than that of compound 406. The potency of synthetic compounds decreased from compound 406 to 504 to 505. The dephospho-analog (503) was completely inactive.

DISCUSSION

The present findings demonstrate that we have been able to accomplish the synthesis of a lipid A preparation with biological properties identical to those of a natural lipid A of *E. coli* F515 (O8:K27, Re-mutant).

There are marked differences between compounds 506 and 406 in regard to endotoxic activities such as pyrogenicity and leukopenia-inducing activity, activities to prepare and provoke the local Shwartzman reaction in rabbits, and, though less specific to LPS and lipid A, lethal toxicity on chicken embryos. In contrast, smaller differences were noted between compounds 506 and 406 concerning other biological activities. These include activation of the horseshoe crab amoebocyte lysate clotting enzyme cascade and the lethal toxicity in galactosamine-loaded mice.

The finding that compound 406 exhibited high lethal toxicity in galactosamine-loaded mice, as did compound 506 and F515 lipid A, but was nontoxic in chicken embryos, suggests that the lethal toxicity noted with mice sensitized by loading with galactosamine may not reflect the lethality in normal mice. We wanted to examine the lethal toxicity of synthetic compounds in normal mice but could not do so because of their limited availability. Thus, a comparison was made with toxicity and decrease in body weight of normal mice. In this assay, the difference of toxicity between compounds 506 and 406 was less obvious than in the assay with chicken embryos. In this concentration, recent studies of Baracos et al. (1) and Goldberg et al. (7) reveal that a severe loss of body mass during fever and infections results from extensive protein degradation in skeletal muscle that was strongly stimulated by leukocyte pyrogen (interleukin I) and prostaglandin E_2.

It appears that the acyloxyacyl groups which are present in compound 506 but absent in compound 406 play a very important role in exhibiting full endotoxic activities of LPS and lipid A. As described in a separate paper (N. Kasai, S. Arata, J. Mashimo, K. Okuda, Y. Aihara, S. Kotani, H. Takada, T. Shiba, and S. Kusumoto, Biochem. Biophys. Res. Commun., in press), there were distinct differences between compounds 506 and 406 in their in vitro antigenicity. In brief, among test synthetic compounds, compound 506 exhibited serological reactivity that was practically indistinguishable from that of F515 lipid A in the reaction with conventional rabbit and mouse antibodies and three monoclonal antibodies against lipid A, while compound 406 had a pattern of reactivities significantly different from those of compounds 506 and F515 lipid A.

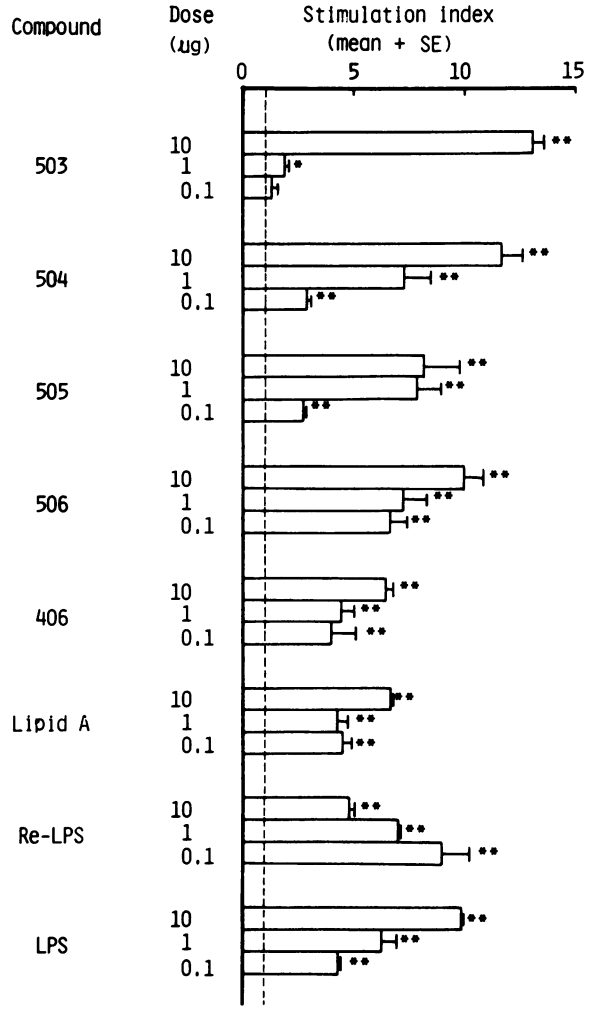

FIG. 7. PBA of BALB/c mouse splenocytes by synthetic lipid A, its analogs, and reference natural products. Doses are shown in micrograms per 6×10^6 cells. The number of anti-TNP-SRBC hemolytic PFC (mean ± SE) in control cultures was 85.6 ± 7.6 per 6×10^6 cells.

Comparisons of the biological activities of monophosphate analogs (504 and 505) with those of the parent molecule (506) revealed that activities generally decreased from the 1,4'-bisphosphate compound (506) through the 4'-monophosphate analog (504) to the 1-monophosphate analog (505) with exception for the mitogenic effects on splenocytes of BALB/c *nu/nu* and C3H/HeN mice. The dephospho-compound (503) was found to be active only in assays of PBA of murine splenocytes and enhanced human PMNL migration. In this connection, it should be noted that compounds 504 through 506, F515 lipid A, and F515 Re-LPS were subjected to in vivo and in vitro assays as TEA salts and therefore in more or less soluble form. Macroscopic inspection shows that the TEA salt of 506 is completely soluble in various assay media and those of 504 and 505 are

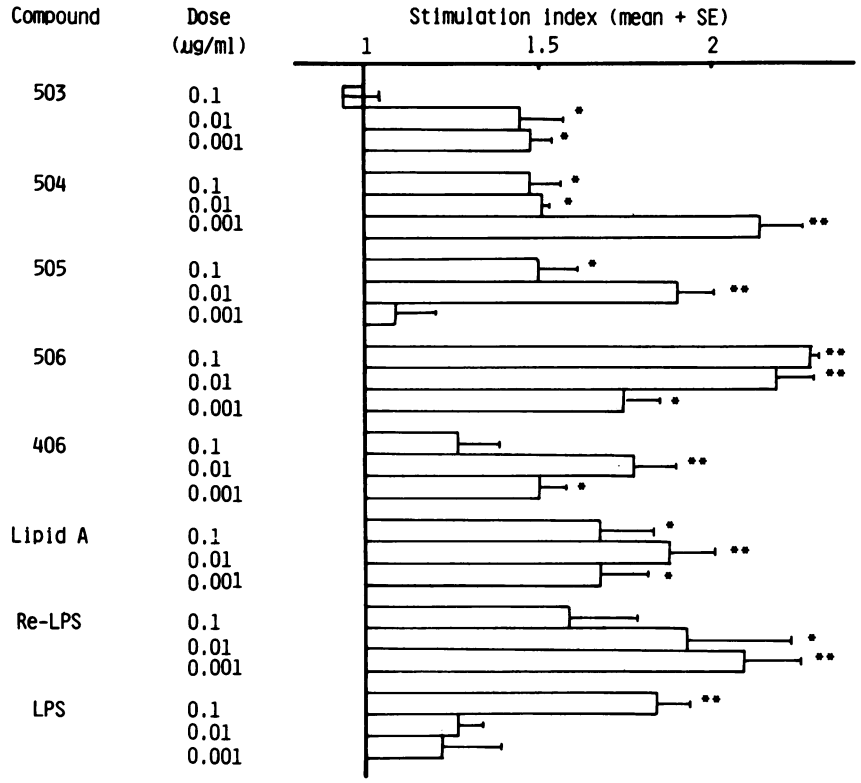

FIG. 8. Enhancement of human PMNL migration in response to synthetic lipid A, its analogs, and reference natural products. The number of PMNL per oil immersion field (mean ± SE) after the reaction for 2 h at room temperature: 0.1 μg of equivalent diluent, 14.7 ± 1.8; 0.01 μg of equivalent diluent, 15.0 ± 1.2; 0.001 μg of equivalent, 13.7 ± 1.8. Stimulation indices (mean ± SE) of positive controls were as follows: *N*-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine (10^{-6} M), 5.55 ± 0.09; a 1:10 dilution of a fresh human serum activated by *S. enteritidis* LPS-B, 7.63 ± 0.68.

slightly less soluble, but no differences cannot be recognized between the latter two compounds. The number or position of substituted phosphate groups in test compounds may cause significant differences in the physical state of the molecule in solvent; in turn, differences in physical properties may have different influences on the efficacy of expression of their inherent biological activities in different assay systems. The dephospho-compound (503), unlike other synthetic compounds, could not be converted to TEA salt and

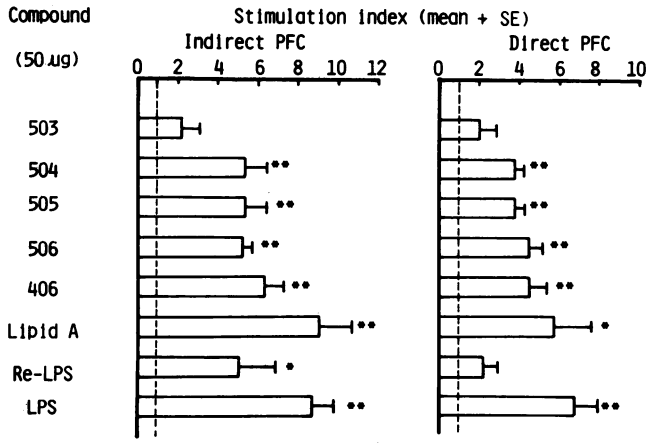

FIG. 9. Adjuvant activity of synthetic lipid A and its analogs to increase anti-SRBC hemolytic PFC number in the spleen of BALB/c mice by intraperitoneal administration together with SRBC compared with those of reference natural products. The dose size was 50 μg of test compound and 2×10^7 SRBC. The numbers of direct and indirect anti-SRBC hemolytic PFC (mean ± SE) in the control mice receiving SRBC alone were 69.4 ± 14.4 and 20.0 ± 20.0 per 10^6 cells.

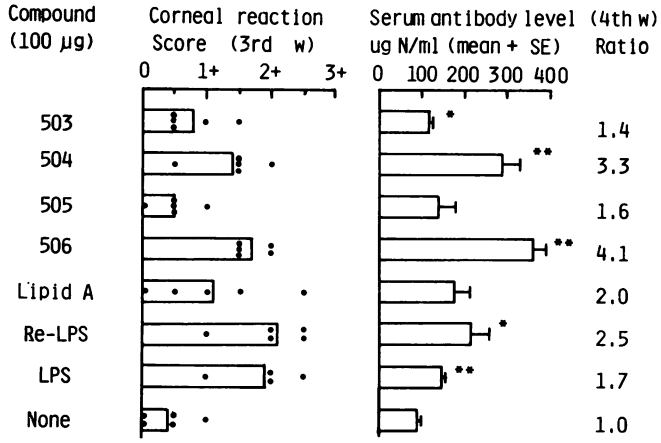

FIG. 10. Adjuvant activities of synthetic lipid A, its analogs, and reference natural products to induce DTH and to raise antibody level in serum to ovalbumin in guinea pigs. The dose size was 100 μg of test compound and 1 mg of ovalbumin per animal. For the corneal reaction, each dot represents the score (arbitrarily graded with a maximum of 3+) for an individual animal, and each column shows the mean score. Antibody level in serum was expressed as micrograms of antibody nitrogen per milliliter of serum and also as ratios of antibody content in each test group to that in the control group.

TABLE 8. Cytostatic and cytocidal effects on L-929 cells of sera from BCG-primed mice, elicited by intravenous injection of synthetic lipid A, its analogs, and reference natural products

Compound	Dose (μg)	Cytostatic action (ED_{50})[a]	Cytocidal action of 1:100 serum	
			No. of dead cells/total[b]	Grade[c]
503	10	<20	17/417	(−)
504	10	1,644	NC[d]	(++)
	2	242	NC	(++)
505	10	560	NC	(++)
	2	<69	5/189	(−)
506	10	2,692	NC	(++)
	2	1,721	NC	(++)
406	10	2,198	NC	(++)
	2	975	NC	(++)
Lipid A	10	3,981	NC	(++)
	2	4,764	NC	(++)
Re-LPS	10	4,753	NC	(++)
	2	584	NC	(++)
LPS	10	3,681	NC	(++)
	2	2,831	NC	(++)
None		<20	5/285	(−)

[a] Reciprocal of the final dilution of test serum that results in 50% inhibition of thymidine uptake of L-929 cells.

[b] Number of dead cells/number of viable and dead cells in 0.1 mm^3 of test specimen, determined by the conventional trypan blue exclusion test.

[c] Grades of killing: ++, intense killing accompanied by extensive destruction of L-929 cells; −, no significant damage to the cells.

[d] NC, Not countable because of extensive destruction of cells with only a few intact cells.

solubilized in this way. This fact may explain the inactivity of compound 503 in almost all of the assay systems.

It may be pertinent to add some comment on the pathway through which human serum complement was activated by compound 506 and natural reference products. There was controversy or confusion concerning the pathway which bacterial LPS activated. This is due to complex chemical composition and considerable variations among species and even strains in the chemical structures of the molecules. However, it has become apparent that a lipid A portion activates the complement via the classical pathway, while a polysaccharide moiety of LPS activates the alternative pathway of the complement (19). The present data indicating that the ability of compound 506 to activate human serum complement was almost completely blocked by the presence of EGTA provide additional and conclusive proof that the lipid A portion causes the activation of complement via the classical pathway. The finding that the activation of complement by *E. coli* O55:B5 LPS was almost completely blocked by addition of EGTA seems to be in conflict with the general view that the activation of the alternative pathway is a property common to LPS from various gram-negative bacteria. This might be associated with the fact that the ability of the LPS preparation used in the present study to activate human serum complement was unexpectedly weak.

Compound 506 exhibited definitely stronger activities than the natural counterpart, F515 lipid A, in some assays, such as those for pyrogenicity, leukopenia-inducing activity, and lethality in galactosamine-loaded mice and the *Limulus* test. A plausible explanation for this finding would be that the F515 lipid A specimen used in this study as a reference standard is a mixture of 1,4′-bisphosphorylated and 4′-monophosphorylated compounds in approximately equal amounts with other minor components, as analyzed by thin-layer chromatography, and the 1,4′-bisphosphate compound (506) tends to be more potent than the 4′-monophosphate analog (504) in the above assays. However, the above statement on relative potencies of compound 506 and F515 lipid A cannot be applicable to the other activities, for example, the mitogenicity and adjuvant activity in mice.

ACKNOWLEDGMENTS

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