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Low Endotoxic Activities of Synthetic *Salmonella*-Type Lipid A with an Additional Acyloxyacyl Group on the 2-Amino Group of β(1-6)Glucosamine Disaccharide 1,4′-Bisphosphate

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A synthetic lipid A (*Salmonella* type, compound 516), β(1-6)-linked D-glucosamine disaccharide 1,4′-bisphosphate, with three acyloxyacyl groups and one hydroxyacyl group, i.e., (*R*)-3-hexadecanoyloxytetradecanoyl, (*R*)-3-hydroxytetradecanoyl, (*R*)-3-dodecanoyloxytetradecanoyl, and (*R*)-3-tetradecanoyloxytetradecanoyl groups at the 2-amino, 3-hydroxyl, 2′-amino, and 3′-hydroxyl groups, respectively, was less biologically active than the synthetic *Escherichia coli*-type lipid A (compound 506), which has only two acyloxyacyl groups at the 2′ and 3′ positions and is substituted with a (*R*)-3-hydroxytetradecanoyl group at the 2-amino group. Compound 516 exhibited considerably weaker pyrogenic and leukopenic activity than compound 506, and it scarcely prepared rabbit skin for the Shwartzman reaction and lacked lethal toxicity on chicken embryos, although its lethal toxicity in galactosamine-loaded mice was as strong as that of compound 506. Compound 516 was also less active than compound 506 or natural *E. coli* lipid A (from Restrain F515) in other biological test systems, such as the *Limulus* test, stimulation of macrophages and lymphocytes, and interferon-inducing activity but not for interleukin-1 induction or complement activation. This observation suggests that there is an optimal number of acyloxyacyl groups on the glucosamine backbone for producing the biological activities of lipid A, especially the endotoxic activities. The 4′-monophosphate analog (compound 514) of compound 516 in general had significantly weaker activity than compound 516 in the above assays, most probably because of its greater hydrophobicity and consequently lower solubility in assay systems. Bacterial R595 lipid A derived from *S. minnesota* Re-mutant, which is a mixture of compounds 516 and 506, their 4′-monophosphate analogs and other compounds, exerted intermediate degrees of activity between compounds 506 and 516 in the various test systems employed.

We (20) previously reported the synthesis of a lipid A with biological activities comparable to those of a natural lipid A, which is responsible for the endotoxic and most of the other biological activities of bacterial lipopolysaccharides (LPS) (7, 21, 38). A synthetic compound (compound 506), β(1-6)-linked D-glucosamine disaccharide 1,4′-bisphosphate, having two (*R*)-3-hydroxytetradecanoyl groups, the (*R*)-3-dodecanoyloxytetradecanoyl, and the (*R*)-3-tetradecanoyloxytetradecanoyl group at 2-amino, 3-hydroxyl, 2′-amino and 3′-hydroxyl groups, respectively, which was prepared according to the proposed structure of lipid A from an *Escherichia coli* Re-mutant (F515) (10, 11; Fig. 1A), exhibited full biological activities identical to or sometimes stronger than those of the reference natural products, F515 lipid A and *E. coli* O55:B5 LPS.

The chemical fine structure of lipid A varies among different bacterial species (21, 29). Therefore, we wanted to examine the influences of the differences in chemical fine structures on biological activities of lipid A's. Difficulties encountered in the use of natural lipid A for the analyses are that the substances are always mixtures of several components with different fine structures, which may be originally present in native LPS of bacterial cell surface layers, or artificially formed during preparation procedures. Thus, the synthetic approach providing chemically and consequently biologically homogenous preparations should have great advantages in analyses of structure-activity relationships of lipid A's.

Among the lipid A's of various bacterial species, those from *Salmonella* spp., particularly the *Salmonella minnesota* Re-mutant (strain R595), have been most extensively studied both chemically and biologically since the early stage of lipid A research (7, 21, 38). Indeed, the backbone structure of lipid A [1,4′-bisphosphorylated β(1-6)-disaccharide of D-glucosamine] was first established by using LPS of the *S. minnesota* Re-mutant R595. However, its fine structure was not clarified until Rietschel et al. (30, 31, 33) postulated the chemical structure of *S. minnesota*-type lipid A in 1984 (Fig. 1B). The unique structural feature of *Salmonella* lipid A is the presence of hexadecanoic acid (palmitic acid) and polar substituents on the phosphate moieties, i.e., 4-aminoarabinose and phosphorylethanolamine, which are not present in *E. coli*-type lipid A. However, analytical data revealed that these three substituents are not present in stoichiometric amounts, indicating that the *Salmonella* lipid A preparations subjected to analysis are heterogenous.

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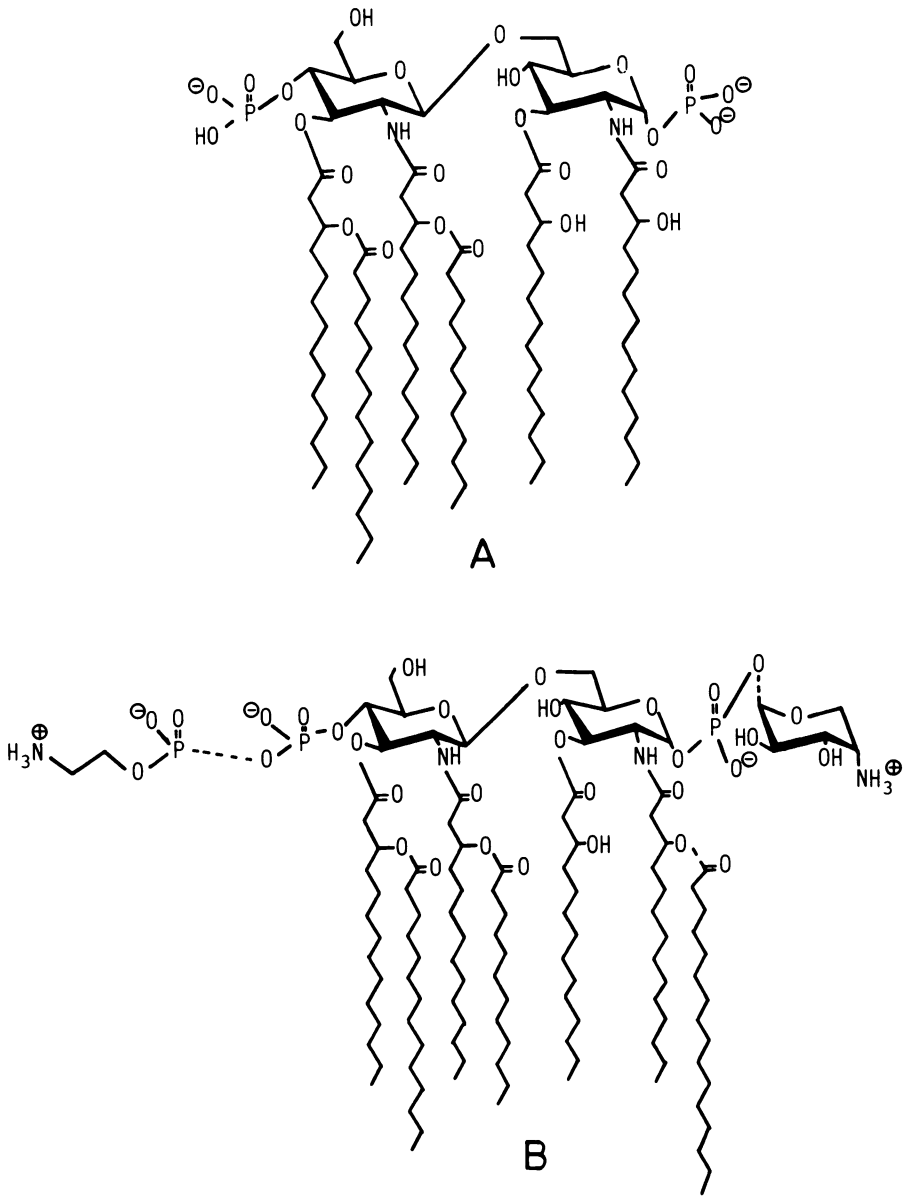


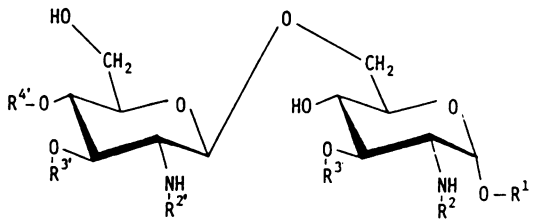
FIG. 1. Proposed chemical structure of natural lipid A's. (A) *E. coli*-type lipid A based on the reports of Imoto et al. (11, 12); (B) *Salmonella*-type lipid A based on the reports of Rietschel et al. (30, 31), Seydel et al. (33), and Strain et al. (34). Dotted lines indicate possible variations.

Thus, we examined the endotoxic and related biological activities of synthetic preparations whose structure corresponds to the components of *Salmonella*-type lipid A. The presence of polar substituents was found to be not essential for the endotoxic activity from our previous study on a synthetic counterpart of *E. coli*-type lipid A (20) and from comparative analyses of a number of natural lipid A preparations, including those of *Salmonella* (29). Therefore, we investigated the effects of the additional hexadecanoyl residues at the 2-amino group of the glucosamine disaccharide backbone, which characterize *Salmonella*-type lipid A. This type of compound containing the complete set of seven fatty acids (compound 516) was thus synthesized.

In the present paper, we described the endotoxic and related biological activities of *Salmonella*-type lipid A, compound 516 and its 4′-monophosphate derivative (compound 514) as compared with those of *E. coli* type lipid A, compound 506 and a natural lipid A prepared from *S. minnesota* Re-mutant (R595) in a number of in vivo and in vitro assay systems. A large number of biological activities were investigated in view of the possibility that synthetic lipid A preparations are expected to be used as a reference standard in a variety of immunobiological assay systems, because of their homogeneity and reproducibility.

MATERIALS AND METHODS

Animals. BALB/c, C57BL/6, DBA/2, C3H/HeN and ICR mice were purchased from Charles River Japan, Osaka, Japan. ICR mice were also supplied by Shizuoka Experimental Animal Co., Hamamatsu, Japan. BALB/c *nu/nu* and C3H/HeJ mice were obtained from Clea Japan, Osaka, Japan, and Jackson Laboratory, Bar Harbor, Maine, through the courtesy of Hajime Kawasaki (NRI Lifescience, Kanagawa,



Compound	$R^{3'}$	$R^{2'}$	R^3	R^2	$R^{4'}$	R^1
516	C_{14}-O-(C_{14})	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-O-(C_{16})	P	P
514	C_{14}-O-(C_{14})	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-O-(C_{16})	P	H
506	C_{14}-O-(C_{14})	C_{14}-O-(C_{12})	C_{14}-OH	C_{14}-OH	P	P
406	C_{14}-OH	C_{14}-OH	C_{14}-OH	C_{14}-OH	P	P

FIG. 2. Chemical structure of synthetic *Salmonella*-type and *E. coli*-type lipid A's and a 4'-monophosphate analog of the former. 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; C_{14}-O-(C_{16}), (*R*)-3-hexadecanoyloxytetradecanoyl.

Japan), respectively. Domestic Japanese white rabbits and Hartley albino guinea pigs were obtained from Nihon Rabbit Co., Osaka, Japan.

Test materials. (i) Synthetic lipid A's and analogs. Compounds 516 and 514 (triethylammonium salt; Fig. 2) were synthesized as described in a separate report (S. Kusumoto, H. Yoshimura, M. Imoto, and T. Shiba. Manuscript submitted for publication). Briefly, the compounds were prepared essentially by the same strategy described previously for the synthesis of compound 506, *E. coli*-type lipid A (12). Thus, coupling of two monosaccharide components, i.e., 2-deoxy-4-diphenylphosphono-3-*O*-[(*R*)-3-tetradecanoyloxytetradecanoyl]-6-*O*-(2,2,2-trichloroethoxycarbonyl)-2-(2,2,2-trichloroethoxycarbonyl-amino)-α-D-glucopyranosyl bromide and allyl 3-*O*-[(*R*)-3-benzyloxytetradecanoyl]-2-deoxy-2-[(*R*)-3-hexadecanoyloxytetradecanoylamino]-α-D-glucopyranoside, afforded a β(1-6)-linked disaccharide. The compound was then acylated at the 2'-amino position with (*R*)-3-dodecanoyloxytetradecanoic acid and phosphorylated at the glycosidic position with dibenzyl phosphorochloridate stepwise after removal of the respective protecting groups. Hydrogenolytic deprotection (palladium followed by platinum) yielded the 1,4'-bisphosphate, which was purified to homogeneity by means of silica-gel column chromatography. Washing with cold, diluted hydrochloric acid, followed by lyophilization from dioxane gave the free acid form of the bisphosphate (compound 516) as a colorless powder. Hydrogenolysis without the 1-phosphorylation step yielded the 4'-monophosphate (compound 514), which was purified in a similar way as that described above.

The purities and structures of all the key synthetic intermediates were confirmed by means of thin-layer chromatography, nuclear magnetic resonance, and elementary analyses. Thin-layer chromatography was performed on precoated silica gel plates (DC-Alufolien, Kiselgel 60 F_{254}; Merck & Co., Inc., Rahway, N.J.), and the spots were visualized by heating on a hot plate after spraying anisaldehyde-sulfuric acid reagent. Each compound showed a single spot with the following R_f value; compound 516, R_f 0.37 and compound 514, R_f 0.83 (chloroform-methanol-water-triethylamine, 10:5:1:0.05, vol/vol). The results of elementary analyses were also satisfactory. Found for compound 516: C, 62.98; H, 10.03; N, 1.37% (calculated for $C_{110}H_{208}N_2O_{26}P_2 \cdot 3H_2O$: C, 63.19; H, 10.31; N, 1.34%). Found for compound 514: C, 66.67; H, 10.60; N, 1.38% (calculated for $C_{110}H_{207}N_2O_{23}P \cdot 1.5H_2O$: C, 66.60; H, 10.67; N, 1.41%).

Compound 506 and compound 406, the latter of which was a synthetic counterpart of a biosynthetic lipid A precursor Ia (5) and whose biological activities were examined previously (6, 13, 18, 35) served as synthetic reference compounds except in a few cases in which comparison was not possible due to their very limited availability.

(ii) Reference natural products. Lipid A specimens from the *S. minnesota* Re-mutant (strain R595) were prepared as described previously (2). Analyses with thin-layer chromatography on Kieselgel 60 (Merck) with developing solvent consisting of chloroform–methanol–water–0.02 M EDTA (100:50:7:4, vol/vol) revealed that this specimen was a mixture of at least eight components, among which the major two components (together approximately 60% of the total components) gave R_f values corresponding to compounds 506 and 516, respectively. Two of the minor components seemed to be 4'-monophosphoryl compounds whose R_f values were close to those of compounds 504 and 514, respectively. An *E. coli* F515 lipid A specimen (a gift of Chris Galanos, Max-Planck-Institut für Immunbiologie, Freiburg, Federal Republic of Germany) and an LPS preparation obtained from *E. coli* O55:B5 (lot no. 203; List Biological Laboratories, Inc., Campbell, Calif.) served as other reference products. The latter material was mainly used to check the validity of the assay system.

Both synthetic and natural products were dissolved at a concentration of 2 mg/ml in 0.1% (vol/vol) triethylammonium aqueous solution. While compounds 506 and 406 as well as the three natural products were clearly dissolved at least macroscopically, compound 516 gave a slightly turbid but not spontaneously sedimentable "solution". Compound 514, on the other hand, could be finely suspended but hardly dispersed to give a stable suspension. The stock solutions

were kept at 4°C and appropriately diluted with distilled water, physiological saline (saline), or cell culture medium before use for assays except in the following tests. For analysis of pyrogenicity and leukopenia-inducing activity in rabbits and preparatory activity for local Shwartzman reaction in rabbits, the above stock solution was diluted with an equal volume of an aqueous solution (2 mg/ml) of bovine serum albumin (BSA; A-7511; Sigma Chemical Co., St. Louis, Mo.) and then diluted with saline. The BSA specimen was shown to be nonpyrogenic at a dose contained in the test specimens, 31.6 μg/kg of body weight.

Lethal toxicity in galactosamine-loaded mice. By the method of Galanos et al. (4), 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 (PBS; 1/100 M [pH 7.5]), followed immediately by intravenous injection of the test materials in 0.2 ml of distilled water. The number of mice that died due to intoxication was counted over a 24-h period; the 50% lethal dose (LD_{50}) was calculated for each group by the method of Kärber (14).

Lethal test in chicken embryos. This test was performed by the method of Finkelstein (3). Briefly, chicken embryos (11 days old, White Leghorn, ISA Babcock-B300; Murayama Poultry Farm, Kagawa, Japan) were injected intravenously with 0.1 ml of the test solutions diluted with saline, and observed for 48 h to determine the LD_{50}.

Pyrogenicity. Japanese domestic rabbits, each weighing 2 to 2.5 kg, were injected intravenously with indicated doses of the test specimens in 5 ml of saline per kg of body weight. Based on the experience in a previous study (20) in which a continuous measurement was made of the rectal temperature of the rabbits injected with compound 506, its analogs, or reference natural specimens for a 12-h period, the measurement in the present study was simplified to determine the rectal temperature of each rabbit every 30 min between 1 and 1.5 h and between 2.5 and 4 h after the injection of test materials for the first- and second-phase temperature, respectively. An increase of rectal temperature of more than 0.6°C was regarded as a positive febrile response.

Leukopenia-inducing activity. Peripheral blood leukocytes were determined as described previously (20) in Japanese domestic white rabbits (weight, 2 to 2.5 kg) at 30-min intervals after the intravenous injection of test materials until the number became normal (usually 2 h after the injection). A 33% decrease in the number of circulating blood leukocytes over the value before injection of test materials was regarded as definite leukopenia.

Preparative activity for local Shwartzman reaction. The local Shwartzman reaction was tested in Japanese white rabbits (female, weighing ca. 3 kg each) by a conventional method (35). In brief, the back skin of the rabbits was depilated in the usual manner, and test materials in 0.2 ml of saline were injected intradermally. After 18 h, the animals were injected intravenously with 100 μg of *E. coli* O127:B8 LPS (LPS-W; Difco Laboratories, Detroit, Mich.) per kg of body weight for provocation of the reaction. The skin sites of the preparative injection were examined for hemorrhagic reactions 20 to 24 h after the LPS elicitation.

Immunoadjuvant activity. (i) Against SRBC. The assay method was as described previously (20). Groups of five BALB/c mice (male, 8 weeks old) were immunized by intraperitoneal injection of 5×10^6 sheep erythrocytes (SRBC) with 50 μg of test materials (PBS alone in control). One week later, anti-SRBC antibody-forming cells were measured by the direct hemolytic plaque-forming assay in a Cunningham chamber. Immunoadjuvant activity of test materials was expressed as the stimulation index, the ratios of the number of plaque-forming cells (PFC) per spleen and per 10^6 cells in each test group to those in the control group.

(ii) Against BSA. Groups of eight BALB/c mice (male, 5 to 6 weeks old) were immunized by subcutaneous injection of 100 μg of BSA (fraction V, Sigma) with 100 μg of a test adjuvant, both of which were incorporated in liposomes (32) consisting of L-α-phosphatidylcholine dimyristoyl (purity, approximately 98%; Sigma), cholesterol (99+%; Sigma), and dicetyl phosphate (Sigma) in a molar ratio of 2:1.5:0.22. The mice received the second injection of BSA alone 28 days after the primary immunization. Five days after the booster, the mice were bled from an inferior ophthalmic vein to obtain serum specimens. Antibody determination was carried out by the passive hemagglutination method and enzyme-linked immunosorbent assay as described previously (25a).

Induction of serum factor cytocidal to L-929 cells in BCG-primed mice. Serum test specimens were obtained by the methods of Carswell et al. (1) with minor modification. Briefly, groups of ICR mice (female, 5 weeks old; Charles River Japan) 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). Each group received an intravenous injection of 0.4, 2, or 10 μg (in 0.2 ml of saline) of the 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 then pooled for each group. The cytocidal effect of test serum specimens was determined by the methods described by Matthews (22) with slight modification. In brief, each well of a 96-well flat-bottomed microculture plate (model 430247; Corning Glass Works, Corning, N.Y.) was filled with 100 μl of L-929 cell suspension (5×10^4 cells, NCTC clone 929, kindly supplied by Research Laboratories, Dainippon Pharmaceutical Co., Osaka, Japan) in Eagle minimum essential medium (Research Foundation for Microbial Diseases, Osaka University, Osaka, Japan) containing penicillin G (100 U/ml) and streptomycin (100 μg/ml), which was supplemented with 10% fetal calf serum (FCS; Flow Laboratories Inc., McLean, Va.). The L-929 cells were cultured for 24 h at 37°C under 5% CO_2 in air (in the following experiments, the cell culture was carried out under the same gaseous environment unless otherwise described) and added with 0.4 μg of actinomycin D (Sigma) per well in 10 μl of Eagle minimum essential medium to inhibit cell growth. An appropriately diluted test serum specimen (100 μl) was then added to each of the wells. After an additional 24-h incubation, the supernatant containing nonadherent cells was carefully removed by gentle aspiration. The cells adhering to the wells were fixed with 5% Formalin and stained with crystal violet. The intensity of staining of the adherent cells was determined by measuring the optical density at 595 nm with a micro enzyme-linked immunosorbent assay autoreader (Titertec Multiscan MC; Flow Laboratories).

Induction of IFN-α/β in mice pretreated with *Propionibacterium acnes*. Groups of ICR mice (female, 10 weeks old; Charles River Japan) were treated by intraperitoneal injection of 1 to 1.5 mg of heat-killed *P. acnes* (in 0.2 ml of PBS). Several days after the treatment, primed mice were intravenously injected with appropriate doses of test material in 0.2 ml of PBS. Blood samples were drawn from the heart 2 h after the eliciting injection, and the levels of interferon (IFN)

in serum were assayed by measuring the capacity to inhibit the cytopathic effect of vesicular stomatitis virus on L-929 cells (26). Titers were expressed as international reference units, with National Institutes of Health reference IFN.

***Limulus* test.** The *Limulus* test was performed by the conventional gelation method with Pre Gel, an amoebocyte lysate of *Tachypleus tridentatus* (Seikagaku Kogyo, Tokyo, Japan), and colorimetric methods, Toxicolor Test (Seikagaku Kogyo) and ES-Test (Seikagaku Kogyo). Details of the Toxicolor Test and ES-Test were described by Obayashi (24) and Obayashi et al. (25), respectively. An LPS specimen from *E. coli* O111:B4 (LPS-W; Difco) was used as a reference standard in these tests.

Activation of human complement. Pooled, fresh adult human serum, kept at −70°C, was incubated with an equal volume of test material solution or suspension appropriately diluted with distilled water. After incubation at 37°C for 1 h, the decrease in total hemolytic activity (median complement hemolytic dose, CH_{50}) was determined by the one-point method (17). The blocking effect on the classical pathway by the addition of 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] plus 5 mM $MgCl_2$ was also determined. In the latter assay, a water-soluble peptidoglycan (SEPS), which was prepared from *Staphylococcus epidermidis* cell walls by using an endopeptidase (16) capable of cleaving cross-linkages to make a polymer of peptidoglycan subunits and was shown to activate human complement system exclusively via the alternative pathway in a previous study (19), was used as a reference.

Stimulation of macrophages in vitro. (i) Glucosamine incorporation. The assay for glucosamine incorporation was done as described previously (20). Oil-induced macrophages obtained from a peritoneal wash of a Hartley albino guinea pig (female, weighing around 500 g) that had received an intraperitoneal injection of 20 ml of sterile liquid paraffin 4 days before were distributed in each well of a 24-well microculture plate (Falcon 3047; Becton Dickinson Labware, Oxnard, Calif.) to make a monolayer, at a cell density of 5×10^6 cells per well per 0.5 ml of RPMI 1640 medium containing penicillin G (100 U/ml) and streptomycin (100 μg/ml) (RPMI medium; Research Foundation for Microbial Diseases, Osaka University, Osaka, Japan) supplemented with 10% FCS. An appropriate dilution (10 μl) of test materials (none in the control) was added to each well, and the cells were cultured for 48 h. During the final 6 h of the culture, the cells were pulsed with 0.1 μCi of D-[^{14}C]glucosamine hydrochloride (New England Nuclear Corp., Boston, Mass.). After completion of the culture, the radioactivity in the trichloroacetic acid-insoluble fraction of each culture was measured by the liquid scintillation method.

(ii) Enhancement of superoxide anion (O_2^-) generation. The superoxide anion generation assay was done as described previously (20). Oil-induced peritoneal macrophages cultivated as described above were exposed to test materials (medium alone in control culture) for 48 h. The cells were preincubated with 1 ml of 50 μM cytochrome *c* (from horse heart, type VI; Sigma) in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer at 37°C for 10 min. Then, 10 μl each of wheat germ agglutinin (4 mg/ml; Sigma) and cytochalasin E (500 μg/ml; Sigma) were added, and the culture was incubated at 37°C for 15 min. The rate of reduction of cytochrome *c* in the reaction mixture was measured by recording the A_{550} to A_{540} with a double-beam spectrophotometer (model 200-20; Hitachi, Tokyo, Japan) and expressed as nanomoles per minute per 10^6 cells (molar absorption coefficient, 19.1×10^3).

(iii) PGE_2 production. The prostaglandin E_2 (PGE_2) production assay was done as described by Nishijima et al. (23). In brief, resident peritoneal cells obtained from normal ICR mice (male, 8 weeks old; Shizuoka Experimental Animal Co.) were washed by and suspended in RPMI medium and then distributed to petri dishes (35-mm diameter; Falcon) at a concentration of 2×10^6 cells per ml per dish. After a 2-h incubation, nonadherent cells were removed by aspiration. After washing of the monolayers three times with Hanks solution, test materials that were appropriately diluted in 1.0 ml of RPMI medium supplemented with 10% FCS were added, and the cultures were kept for 24 h. Culture supernatants were collected, and the content of PGE_2 was measured by radioimmunoassay with the bicyclic PGE_2 assay system (TRK 800; Amersham Japan, Tokyo, Japan).

(iv) IL-1 production. Interleukin 1 (IL-1) production was measured by the method of Vacheron et al. (37) with slight modifications. Exudate cells were collected by peritoneal washing with 10 ml of PBS from DBA/2 mice (female, 8 to 10 weeks old) that were pretreated by intraperitoneal injection of 1 ml of thioglycolate broth (Nissui Pharmaceutical Co., Tokyo, Japan) 3 days before. After two washes, the cells (1.5×10^6 cells per 3 ml per dish) were suspended in RPMI medium supplemented with 10% FCS and distributed in petri dishes (35-mm diameter; Falcon 3001; Becton Dickinson Labware). After a 2-h incubation, nonadherent cells were removed by aspiration. The monolayers were washed twice with PBS, and the indicated doses of test materials in 3 ml of RPMI medium without FCS were added and then cultured at 37°C for 24 h. Culture supernatants were collected, and their IL-1 activity was determined as follows. Thymocytes (1.5×10^6 cells per 0.1 ml per well) of C3H/HeJ mice (male, 4 to 8 weeks old; nonresponders to LPS) were suspended in RPMI medium supplemented with 10% FCS and distributed in a 96-well microculture plate (Corning) (1.5×10^6 cells per 0.1 ml per well). To each well, 0.1 ml of twofold serially diluted culture supernatants and a submitogenic concentration (final concentration, 1 μg/ml) of phytohemagglutinin (HA16, Wellcome Research Laboratories, Beckenham, England) were added, and cells were cultured for 72 h. During the final 6 h of the culture, cells were pulsed with 0.5 μCi of [^{3}H]thymidine (Amersham International, Buckinghamshire, England), and incorporation of thymidine was measured by the conventional scintillation method.

Stimulation of murine splenocytes. (i) Thymidine uptake. Splenocytes (5×10^5 cells) of BALB/c *nu/nu* (male, 8 weeks old) and C3H/HeJ (male, 6 to 8 weeks old) mice were cultured with graded doses of test materials in 0.2 ml of RPMI medium supplemented with 10% FCS for 48 h. In the experiment with splenocytes of C3H/HeJ mice, phytohemagglutinin and concanavalin A (type IV, Sigma) were used as controls for checking the viability, or reactivity of the cells. During the final 24 h of cultivation, cells were pulsed with 0.5 μCi of [^{3}H]thymidine. Thymidine uptake was measured by the conventional scintillation method (35).

(ii) PBA. Measurements for polyclonal B-cell activation (PBA) were made as described previously (35). Briefly, splenocytes (6×10^6 cells) of BALB/c mice (male, 8 weeks old) were cultured with graded doses of test materials in RPMI medium supplemented with 10% FCS. After a 72-h cultivation, the number of hemolytic PFC against 2,4,6-trinitrophenylated SRBC was determined in a Cunningham chamber.

Other procedures. Meticulous precaution was taken to

TABLE 1. Lethal toxicity of synthetic lipid A, its 4'-monophosphate analog, and reference synthetic and natural products in galactosamine-loaded mice

Compound	No. of deaths/no. of mice tested with the following dose (ng)				LD_{50} (ng)
	100	10	1	0.1	
516	5/5	4/5	0/5		5.0
514	5/5	0/5			31.6
506	5/5	3/5	0/5		7.9
R595 lipid A			5/5	0/5	0.3

avoid external contamination of experimental ware and reagents with LPS. In most of the in vitro assays, each determination was made in triplicate cultures to obtain the mean and standard error of the stimulation index (ratios of values in each test culture to those in the respective control culture). Statistical significance of the difference between each test and the respective control was examined by Student's *t* test in most of the assays.

RESULTS

Endotoxic activities. (i) Lethal toxicity. In the assay with galactosamine-loaded mice, compound 516 exhibited definite lethal toxicity; its LD_{50} was similar to that of compound 506 (Table 1). The toxicity of the 4'-monophosphate derivative, compound 514, was about fivefold weaker than that of compound 516. The natural *Salmonella* (R595) lipid A was more toxic than compounds 506 and 516. Regarding lethal toxicity in chicken embryos, compounds 516 and 514 lacked activity at a dose of up to 10 μg per embryo, while compound 506 and R595 lipid A were toxic at 0.1 μg per embryo (data not shown).

TABLE 2. Pyrogenicity and leukopenia-inducing activity of synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and reference synthetic and natural products

Compound and dose (μg/kg)	Febrile response			No. of rabbits showing leukopenia/no. tested[c]
	No. of rabbits positive/no. tested[a]	Change in temp (°C) at peak[b]		
		1	2	
516				
10.0	2/2	0.93	1.38	2/2
1.0	2/2	0.37	0.82	0/2
0.1	2/2	0.41	0.94	0/2
0.01	0/2	−0.62	−0.54	0/2
514				
31.6	2/2	0/54	1.71	0/2
10.0	0/2	−0.13	0.13	0/2
1.0	0/2	−0.09	0.38	0/2
506				
1.0	2/2	1.48	1.55	2/2
0.1	2/3	0.85	1.00	2/3
0.01	2/2	0.78	1.23	2/2
0.001	0/2	0.33	0.48	0/2
R595 lipid A				
1.0	2/2	1.20	1.19	1/2
0.1	2/2	0.40	0.69	1/2
0.01	0/2	−0.65	−0.48	0/2

[a] A positive result was defined as an increase in rectal temperature of more than 0.6°C.

[b] The mean temperature increase in two or three rabbits, irrespective of positive and negative febrile response.

[c] A positive result was defined as a decrease in circulating blood leukocytes over 33% of the value before the injection of test materials.

TABLE 3. Activity of synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and reference synthetic and natural products to prepare rabbit skin for the local Shwartzman reaction[a]

Compound	No. of hemorrhagic sites/no.of sites tested with the following dose (μg/site)						
	80	40	20	10	5	2.5	1.25
516	2/2	0/2	0/2	0/2	0/2	0/2	
514	0/2	0/2	0/2	0/2	0/2	0/2	
506			4/4	4/4	3/4	3/4	1/2
R595 lipid A			4/4	4/4	4/4	4/4	0/2
F515 lipid A			4/4	4/4	4/4	4/4	2/2

[a] Skin sites of rabbits were prepared by intracutaneous injection of the indicated doses of test compounds (in 0.2 ml of saline). At 18 h later, the rabbits received an intravenous injection of 100μg of *E. coli* O127:B8 LPS (in 10 ml of saline) per kg of body weight for provocation of the local Shwartzman reaction.

(ii) Pyrogenicity and leukopenia-inducing activity. Compound 516 induced a definite febrile response in rabbits, but the response was significantly weaker than that of compound 506 (Table 2). Compound 506 at a dose of 0.01 μg/kg of body weight induced a biphasic febrile response, typical of the fever induced by LPS or lipid A, while the animals injected with 0.01 μg of compound 516 per kg of body weight did not exhibit significant fever, and those receiving doses of 0.1 or 1 μg of this compound per kg of body weight did not show a significant peak 1 response. Compound 514 was far less pyrogenic than compound 516, and its minimum pyrogenic dose was 31.6 μg/kg. Concerning leukopenia-inducing activity, compound 516 at a dose of 10 μg/kg induced a positive reaction under the experimental conditions, in which compound 506 induced leukopenia at a dose of 0.01 μg/kg. Compound 514 lacked such activity at doses as high as 31.6 μg/kg. The pyrogenicity and leukopenia-inducing activity of the reference R595 lipid A were intermediate between those of compounds 516 and 506.

(iii) Preparative activity for the Shwartzman reaction. In good agreement with the low pyrogenicity and leukopenia-inducing activity, compound 516 was by far less active in preparing the local Shwartzman reaction than compound 506 and the natural lipid A's (R595 and F515) (Table 3). Thus, for compound 516 doses as high as 80 μg per site were required for preparation, while compound 506 and R595 and F515 lipid A's could prepare the reaction with doses of around 2.5, 2.5, and less than 1.25 μg, respectively. Compound 514

TABLE 4. Activation of the clotting enzyme cascade of *T. tridentatus* amoebocyte lysate by synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and reference synthetic and natural products

Compound	Minimum effective dose for (ng/test)	Colorimetric method equivalent of reference LPS (mg/mg)[a] by:	
		Toxicolor Test	ES-Test
516	0.01	1.50	1.41
514	0.1	0.43[b]	0.45[b]
506	0.01	5.52	5.18
406	0.01	1.98	1.56
R595 lipid A	0.01	3.48	3.06
F515 lipid A	0.01	4.45	3.96

[a] The reference LPS was LPS-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.

[b] Insolubility of the test compound prevented precise estimation.

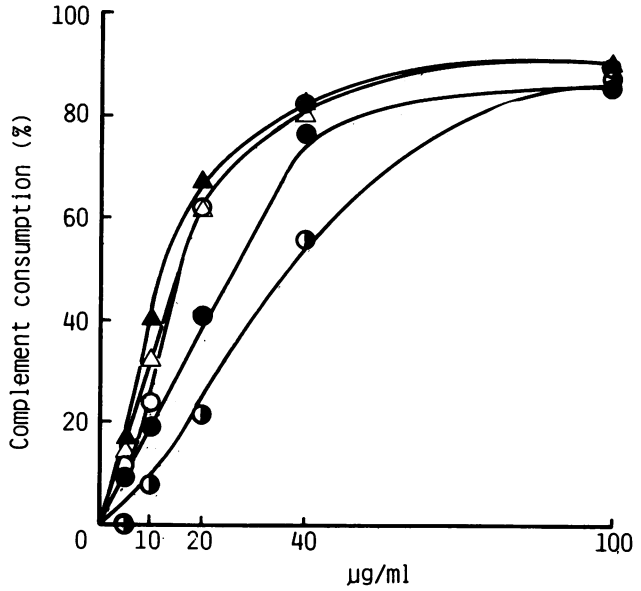


FIG. 3. Activation of human serum complement by synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and reference synthetic and natural products. Symbols: ○, compound 516; ◑, compound 514; ●, compound 506; △, R595 lipid A; ▲, F515 lipid A.

was inactive at a dose of 80 μg/kg, the highest dose examined.

***Limulus* test.** The results obtained by the conventional Pre Gel test indicated that all of the synthetic compounds except compound 514 exhibited potent activity, close to that of natural R595 and F515 lipid A's (Table 4). On the other hand, the Toxicolor Test and ES-Test, the latter being more specific to LPS than the former, revealed considerable differences among the activities of these materials. Namely, compound 516, like compound 406, was slightly weaker than compound 506, whose activity was comparable to or slightly stronger than that of the natural lipid A's, although the

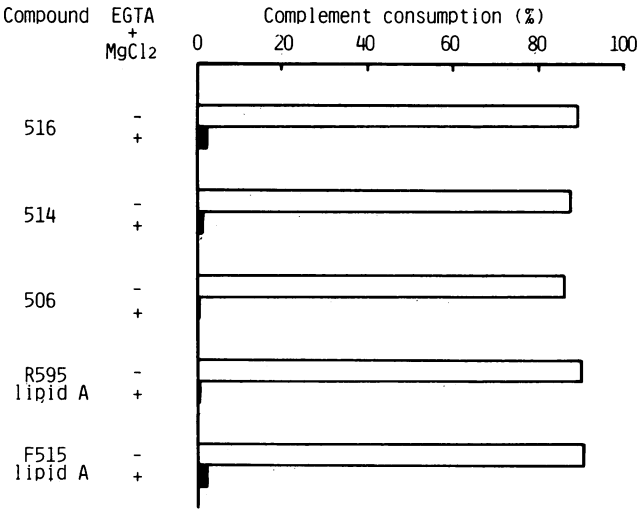


FIG. 4. Blocking effect of the classical pathway by addition of EGTA (10 mM) and $MgCl_2$ (5 mM) on complement activation by synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and reference synthetic and natural products (100 μg/ml).

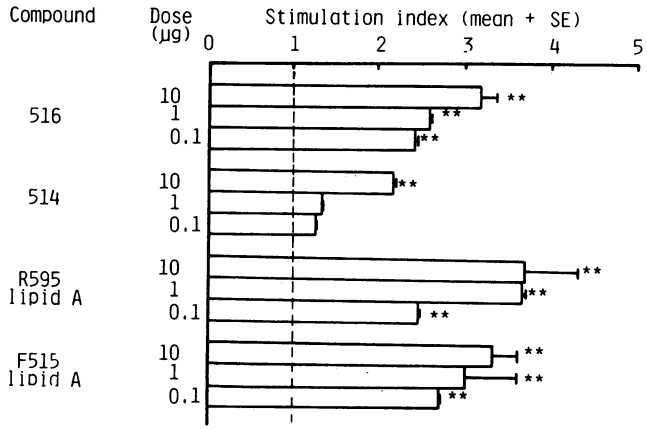


FIG. 5. Effects of synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and reference natural products on glucosamine uptake by guinea pig peritoneal macrophages. Doses are shown in micrograms per 5 × 10^6 cells. The counts of [^{14}C]glucosamine uptake (mean ± standard error[SE]) in control cultures were 232 ± 13 dpm. Results significantly different from the control value ($P < 0.01$) are indicated (**).

values given by these tests differed slightly from assay to assay. The activity of compound 514 was about 1/10 of that of compound 506.

Immunobiological activities. (i) Activation of human complement. Compound 516 activated the complement cascade in human serum in a manner similar to that of R595 or F515

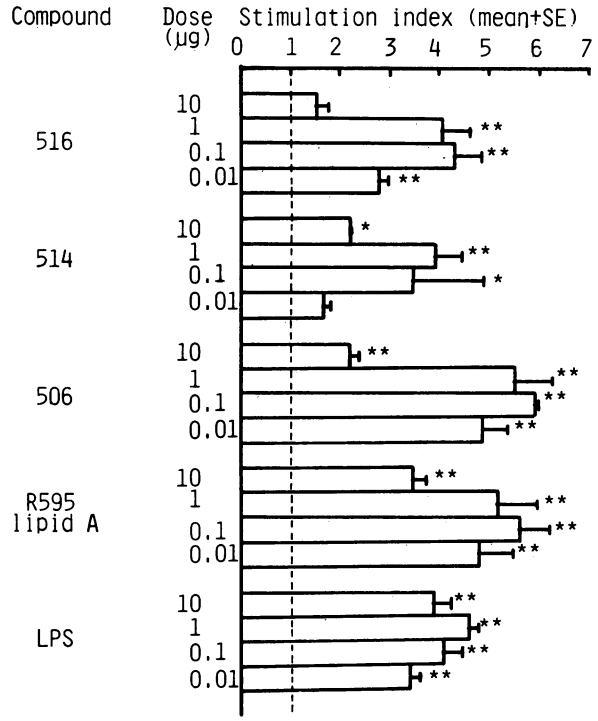


FIG. 6. Effects of pretreatment with synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and reference synthetic and natural products on superoxide anion generation in guinea pig peritoneal macrophages. Doses are shown in micrograms per 5 × 10^6 cells. Superoxide anion generation in control cultures (mean ± standard error[SE]) was 269 ± 52 nmol/min per 10^6 cells. *$P < 0.05$; **, $P < 0.01$.

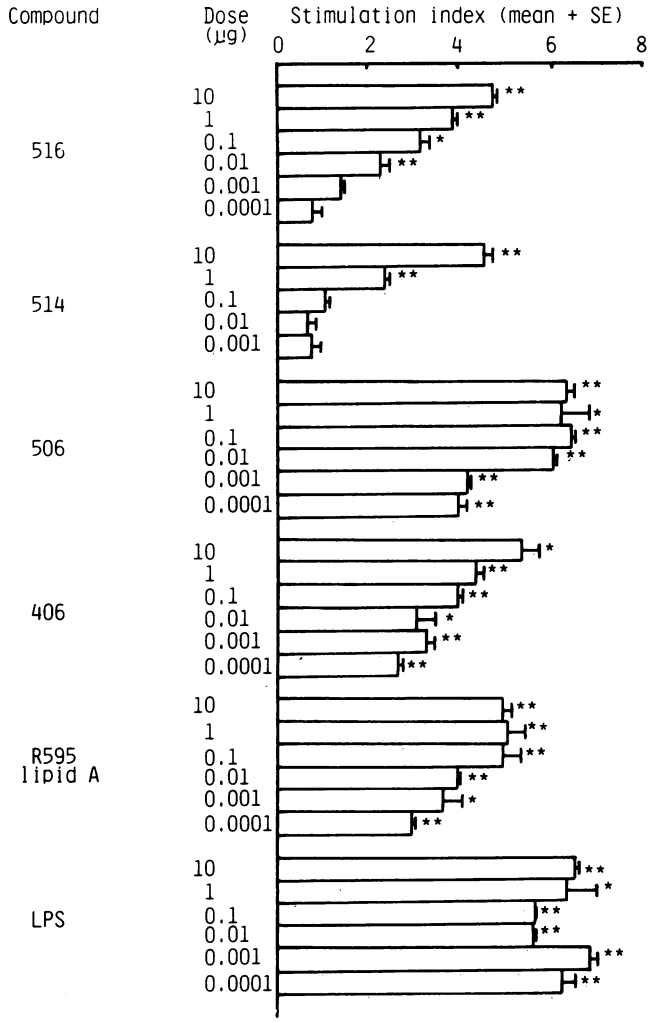


FIG. 7. PGE_2 production by murine resident peritoneal macrophages stimulated by synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and reference synthetic and natural products. Doses are shown in micrograms per 2×10^6 cells per ml. PGE_2 production in control cultures (mean ± standard error[SE]) were 113 ± 8 pg per culture. *, $P < 0.05$; **, $P < 0.01$.

lipid A and more effectively than compound 506 (Fig. 3). Compound 514 was less active than the above materials. We then attempted to assess whether the complement activation by these materials was influenced by blocking the classical pathway by addition of EGTA (10 mM) and $MgCl_2$ (5 mM) to the assay system. Activation of complement hardly occurred with any of the test compounds in the presence of EGTA (Fig. 4) under the experimental conditions in which the activation of complement by SEPS, a polymer of peptidoglycan subunits, was not affected by the blocking action of EGTA (data not shown). This finding indicates that complement activation by the synthetic compounds tested, as well as that by natural lipid A's, proceeds almost exclusively via the classical pathway.

(ii) Stimulation of macrophages. Compound 516 stimulated peritoneal macrophages of guinea pigs and mice in various ways. A significant increase of glucosamine uptake (Fig. 5) and enhanced production of superoxide anion (Fig. 6) were noted with guinea pig macrophages. In murine peritoneal macrophages, this compound also markedly enhanced PGE_2 production (Fig. 7) and IL-1 formation (Table 5); the latter data were representative, but a similar tendency was observed in cultures stimulated with higher doses (1 and 10 μg/ml) of the test compounds (data not shown). Throughout the assays described in the preceding paragraphs, compound 516 was less active than compound 506 and R595 lipid A except for IL-1 production. In the latter assay, compound 516 showed slightly higher activity than the above two reference specimens. The monophosphate derivative, compound 514, was much less active against macrophages than compound 516 in all of the assays. Compound 406 increased PGE_2 production to a greater extent than compound 516 but to a weaker extent than compound 506.

(iii) Activation of murine splenocytes. Compound 516 exhibited strong mitogenic activity on splenocytes from athymic nude mice but had no effect on LPS nonresponding C3H/HeJ mice (Fig. 8). The activity of 516 was considerably weaker than that of the reference products, namely compound 506 and R595 lipid A. Compound 516 also exerted significant PBA activity on murine splenocytes, but as for mitogenicity, the activity was far less than that of compound 506 and R595 lipid A (Fig. 9). R595 lipid A was definitely less active than compound 506 in PBA. Compound 514 had slightly weaker lymphocyte-stimulatory activity than compound 516 in both assays.

(iv) Immunoadjuvant activities in vivo. Compound 516 markedly increased the number of anti-SRBC PFC in spleens of BALB/c mice, which were immunized by intraperitoneal injection of SRBC and compound 516 in PBS, in comparison with that of the control mice receiving SRBC alone. Stimulatory activity of compound 516 in terms of stimulation index was comparable to that of R595 lipid A at a test dose of 50 μg per mouse (Table 6). Compound 514 exhibited only marginal activity in this assay.

TABLE 5. IL-1 production by murine peritoneal macrophages stimulated by synthetic *Salmonella*-type lipid A, its 4'-monophospate analog, and reference synthetic and natural products

Compound	Stimulation index (mean ± SE) at indicated dilution of macrophages culture supernatant[a]			
	1:1	1:2	1:4	1:8
516	14.6 ± 0.6[b]	13.7 ± 1.2[b]	11.4 ± 1.3[b]	12.4 ± 1.1[b]
514	4.7 ± 0.2[b]	2.3 ± 0.1[b]	1.7 ± 0.4	1.7 ± 0.4
506	10.1 ± 1.5[b]	9.8 ± 0.4[b]	11.5 ± 0.2[b]	9.8 ± 1.2[b]
406	9.3 ± 1.5[b]	8.1 ± 0.4[b]	5.2 ± 0.8[b]	4.9 ± 0.4[b]
R595 lipid A	11.8 ± 0.5[b]	10.5 ± 1.1[b]	10.6 ± 1.0[b]	9.5 ± 0.4[b]
None	0.9 ± 0.3	1.9 ± 0.7	0.6 ± 0.1	0.8 ± 0.1

[a] Macrophages (0.5×10^6/ml) from DBA/2 mice were cultured with 0.1 μg of test material per ml for 24 h. The count in control C3H/HeJ thymocyte cultures (with 1 μg of phytohemagglutinin per ml alone) was 1,506 ± 237 dpm.
[b] Significantly different from the respective control ($P < 0.01$).

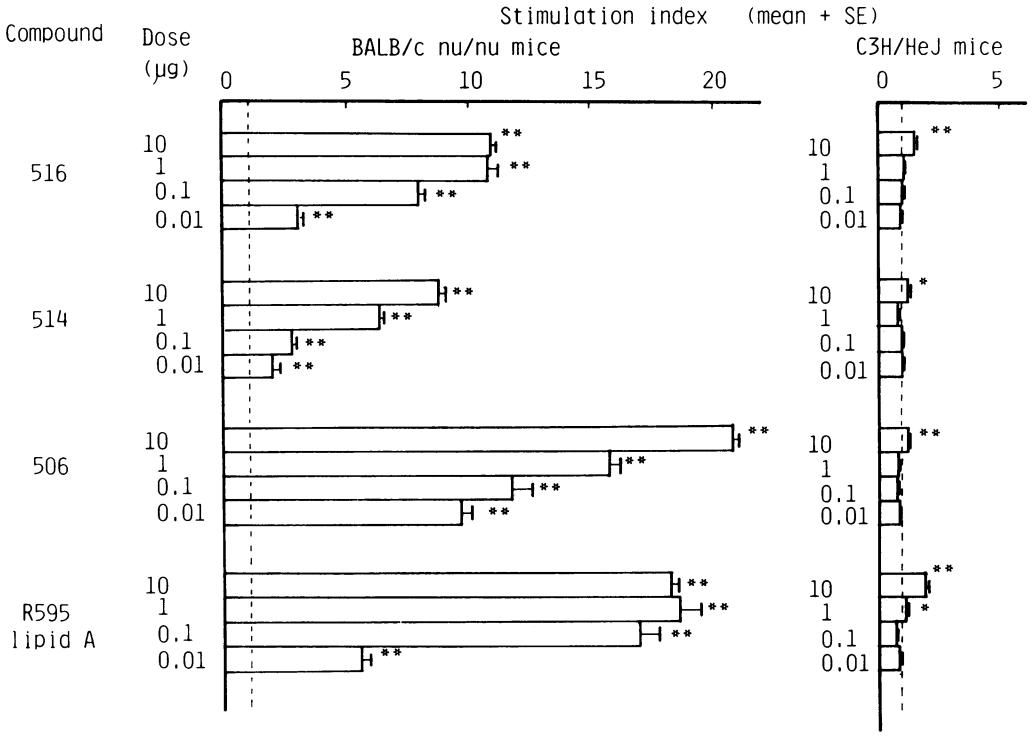


FIG. 8. Thymidine uptake in BALB/c *nu/nu* and C3H/HeJ mouse splenocytes by synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and reference synthetic and natural products. Doses are shown in micrograms per 5×10^5 cells. The stimulation indexes (mean ± standard error[SE]) of phytohemagglutinin (1 μg) and concanavalin A (1 μg) in the experiment with C3H/HeJ mouse splenocytes were 52.7 ± 5.0 and 52.0 ± 2.8, respectively. The counts (mean ± standard error[SE]) in control cultures were 4,132 ± 138 dpm (BALB/c *nu/nu* mice) and 3,319 ± 12 dpm (C3H/HeJ mice). **, $P < 0.01$.

In the system with BSA as the immunogen and liposomes as the administration vehicle, both test synthetic compounds 516 and 514 showed strong adjuvant activity, comparable to that of reference R595 lipid A in enhancement of serum anti-BSA antibody levels of both immunoglobulin G (IgG) and IgA (Table 7). There were no significant differences between the two synthetic specimens. Concerning the IgM level, none of the test materials examined exhibited significant potentiating activity.

(v) Induction of serum factor cytocidal to L-929 cells in

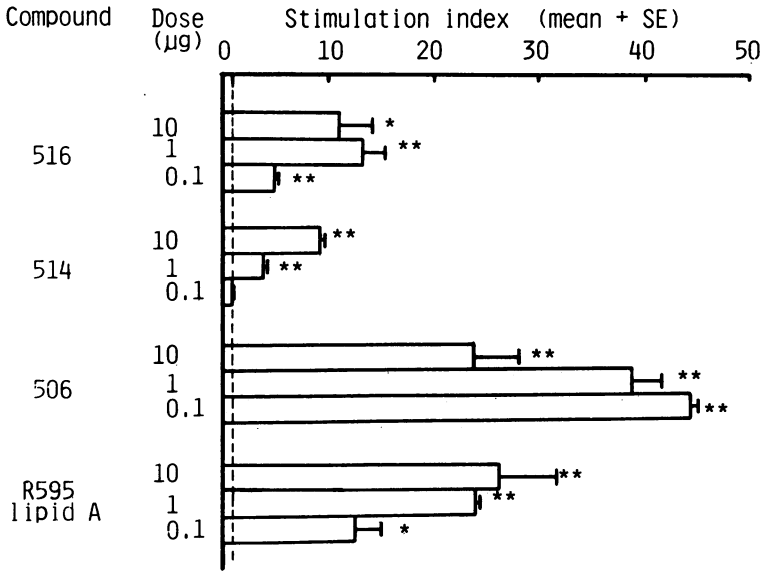


FIG. 9. PBA of BALB/c mouse splenocytes by synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and reference synthetic and natural products. Doses are shown in micrograms per 2.5×10^6 cells. The number of anti-2,4,6-trinitrophenylated-SRBC hemolytic PFC (mean ± standard error[SE]) in control cultures was 32 ± 3 per 2.5×10^6 cells. *, $P < 0.05$; **, $P < 0.01$.

TABLE 6. Adjuvant activity of synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and reference natural products to increase number of anti-SRBC hemolytic PFC in the spleens of BALB/c mice by intraperitoneal injection with SRBC[a]

Compound	No. of PFC (stimulation index) (mean ± SE) per:	
	Spleen	10^6 cells
516	172,300 ± 7,100 (6.63 ± 0.27)[b]	585 ± 9 (5.09 ± 0.08)[b]
514	41,760 ± 2,660 (1.61 ± 0.10)[b]	170 ± 25 (1.48 ± 0.22)
R595 lipid A	141,960 ± 24,780 (5.46 ± 0.95)[b]	549 ± 116 (4.77 ± 1.01)[b]
F515 lipid A	214,270 ± 14,060 (8.24 ± 0.54)[b]	678 ± 80 (5.90 ± 0.70)[b]
None	25,990 ± 2,050 (1.00 ± 0.08)	115 ± 6 (1.00 ± 0.05)

[a] Groups of five BALB/c mice were immunized by intraperitoneal injection of 5 × 10^6 SRBC together with 50 μg of test adjuvant. At 7 days later, the number of anti-SRBC hemolytic PFC in the spleens was determined by a direct method.
[b] $P < 0.01$.

BCG-primed mice. Compound 516 induced a serum factor that exerted powerful cytocidal effects on cultured L-929 cells (a possible tumor necrosis factor) by intravenous injection in BCG-primed mice, although its activity was considerably weaker than that of R595 lipid A when both the effective dose and the strength of cytocidal effects in terms of definitively cytocidal serum dilution were considered together (Fig. 10). Compound 514 was far less active than compound 516.

(vi) Induction of IFNs in *P. acnes*-primed mice. Cumulative results from several experiments are summarized in Table 8. Compound 516 definitely induced serum IFN-α/β, but its ability was considerably weaker than that of compounds 506 and 406; the latter two synthetic compounds powerfully induced IFNs to levels comparable to those induced by F515 lipid A and reference LPS. On the other hand, the monophosphate derivative, compound 514, did not exhibit activity even at the highest dose examined (31.6 μg per mouse).

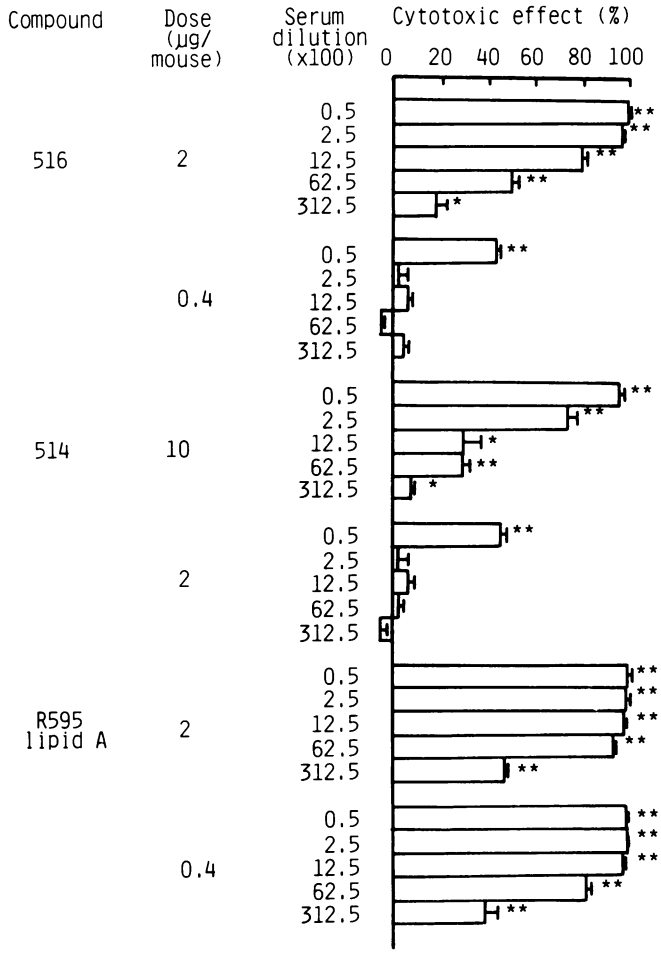


FIG. 10. Cytocidal effects on L-929 cells of serum specimens from BCG-primed mice, elicited by intravenous injection of synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and reference natural product. *, $P < 0.05$; **, $P < 0.01$.

DISCUSSION

This study revealed that a synthetic lipid A (compound 516) carrying three acyloxyacyl groups at C-2, C-2', and C-3' positions, which corresponds to a typical lipid A structure reported to occur in *S. minnesota* (27, 30, 31, 33), exhibited low endotoxic and related biological activities; i.e., lethal toxicity in chicken embryos, pyrogenicity, leukopenia-inducing activity, preparative ability for local Shwartzman reaction, and activity in the colorimetric *Limulus* test. These activities of compound 516 were considerably weaker than those of compound 506, which carried only two acyloxyacyl groups, at the C-2' and C-3' positions. Compound 516, however, exhibited strong lethal toxicity in galactosamine-loaded mice as did compound 506. In this assay system, compound 406 also exhibited definite lethal toxicity (6, 13,

TABLE 7. Adjuvant activity of synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and a reference natural product on anti-BSA antibody production of BALB/c mice by subcutaneous injection with BSA in liposomes[a]

Compound	Passive hemagglutination ratio (2^n) (mean ± SE)	Result of enzyme-linked immunosorbent assay (mean ± SE) for:		
		IgG ratio[b]	IgM (OD_{405} × 100)[c]	IgA (OD_{405} × 100)[c]
516	8.57 ± 0.30[d]	70.19 ± 17.13[d]	150 ± 22	115 ± 8[d]
514	9.71 ± 0.18[d]	60.80 ± 13.96[d]	136 ± 6	114 ± 6[d]
R595 lipid A	9.14 ± 0.51[d]	102.71 ± 39.43[d]	217 ± 44	99 ± 11[d]
None	3.25 ± 0.48	1.00 ± 0.30	208 ± 20	24 ± 8

[a] Groups of eight BALB/c mice were immunized by subcutaneous injection of 100 μg of BSA with 100 μg of test adjuvant in liposomes, and 28 days later they received the second injection of 100 μg of BSA alone in liposomes. Five days after the booster, the anti-BSA antibody level in serum was determined by the passive hemagglutination and enzyme-linked immunosorbent assay methods.
[b] Ratio of the value in each test group to that in the control.
[c] OD_{405}, Optical density at 405 nm.
[d] $P < 0.01$.

TABLE 8. Induction of IFN-α/β in mice pretreated with *P. acnes* by synthetic *Salmonella*-type lipid A, its 4'-monophosphate analog, and reference synthetic and natural products[a]

Compound	Serum IFN-α/β titer (IU/ml of serum) at following dose (μg/mouse) (no. of mice tested)						
	31.6	10	3.16	1	0.316	0.1	0.01
516	1,920 (3)	1,067 (3)	853 (3)	67 (3)			
514	40 (3)	47 (3)					
506			2,560 (3)	800 (4)	1,920 (2)	360 (4)	40 (1)
406		3,413 (3)		4,267 (3)	2,133 (3)	226 (3)	
F515 lipid A		2,560 (2)	3,840 (2)	960 (4)	1,920 (2)	480 (4)	
LPS		1,920 (2)	3,840 (2)	1,710 (3)	3,840 (2)	960 (4)	

[a] ICR mice pretreated with *P. acnes* 1 week earlier were intravenously challenged with test compounds.

18, 35), suggesting that the lethal toxicity in galactosamine-loaded mice does not necessarily reflect the endotoxicity of the compounds, although no definite conclusion can be drawn until a sufficient amount of synthetic compounds becomes available for comparing the lethal toxicities of the compounds in normal mice. Lower activity of compound 516 than natural and synthetic reference products was also observed in the following assays: stimulatory effects on macrophages to increase glucosamine uptake and superoxide anion production (with guinea pig macrophages) and to enhance PGE_2 production (with murine macrophages), lymphocyte-activating effect (mitogenic and PBA activity) in vitro, and tumor necrosis factor and IFN-α/β-inducing activity in BCG- and *P. acnes*-primed mice, respectively, in vivo, although in complement activation and stimulation of murine macrophages to enhance IL-1 release, compound 516 was slightly more effective than compound 506. Compound 516 also had less immunoadjuvant activity in vivo than *E. coli* F515 lipid A, which is a natural counterpart of compound 506 and which has identical or slightly weaker activity than compound 506 (8, 9, 20). Limited availability of compound 506 at present did not permit the direct comparison beween compounds 516 and 506 in the adjuvant activities.

We previously (18, 20, 35) reported that a synthetic lipid A precursor (Ia [4]), compound 406 having no acyloxyacyl groups, exhibited no or very low endotoxic activities; namely, it lacked lethal toxicity in chicken embryos and preparative activity for the local Shwartzman reaction, and it exhibited only weak pyrogenicity, although it exhibited strong lethal toxicity in galactosamine-loaded mice. The other immunobiological activities of compound 406 were also weaker than those of compound 506 except for mitogenic activity. Similar findings were reported by Galanos et al. (6) and Kanegasaki et al. (13). Recently, Galanos et al. (5) showed that a lipid A precursor, Ib, which differs from precursor Ia described above in having one acyloxyacyl group at the C-2 position, exhibited preparative and provocative activities for the local Shwartzman reaction. The activities, however, were slightly weaker than those of F515 lipid A, and its pyrogenicity was as weak as that of precursor Ia.

The above findings suggest that there is an optimal number of acyloxyacyl groups at the β(1-6)glucosamine disaccharide backbone to exert strong endotoxic and related biological activities of lipid A. However, it is not clear at present whether the observed differences in the activities of these 1,4'-bisphosphorylated diglucosamine compounds are derived directly from the differences in chemical structures or result indirectly from the differences in physical (or physicochemical) properties due to structural differences. In this connection, it is necessary to consider whether *S. minnesota*-type lipid A, which is more hydrophobic than that of *E. coli* type by the possession of an additional acyloxyacyl group, can express its inherent biological activities more efficiently by polar substituents, such as 4-aminoarabinose and phosphorylethanolamine, which make the molecule more hydrophilic. In addition, the position of acyloxyacyl groups at the disaccharide backbone might also be important for exhibition of their biological activities, although further experimental evidence is needed to support this assumption.

The biological activity of a natural product derived from *S. minnesota* Re-strain R595 used as a reference in this study was generally higher than that of compound 516 but lower than that of compound 506. This finding is in accordance with the above analytical thin-layer chromatographic data with the specimen, namely it consisted mainly of two structures corresponding to compounds 506 and 516. Seydel et al. (33) and Qureshi et al. (27) also reported similar analytical results with *Salmonella* lipid A. In this connection, we (15) observed that in the reaction with anti-R595 lipid A monoclonal antibodies, compound 516 exhibited highly specific in vitro antigenicity, comparable to that of a natural product, R595 lipid A, and was much stronger than compound 506. This finding does not conflict with the above finding that compound 516 was less bioactive than compound 506, because differences of reactivity between these two synthetic compounds were noted only when a comparison was made with reactivity against special monoclonal antibodies, while conventional antibody raised against the same lipid A could not distinguish the above two compounds.

The 4'-monophosphate analog of compound 516, compound 514, was weaker than compound 516 in all of the assay systems, except immunoadjuvant activity against BSA in mice, which was examined with liposomes as the administration vehicle. This finding is consistent with our previous observation that synthetic lipid A analogs of compound series 400 and 500, lacking a phosphate group at the C-1 position (compounds 404 and 504), exhibited weaker activities than the respective 1,4'-bisphosphate compound (18, 20, 35) and is in accord with the observations by Qureshi et al. (28) and Takayama et al. (36) with bacterial products, i.e., that 4'-monophosphoryl lipid A isolated from the lipid A fraction of *Salmonella typhimurium*, whose chemical structure corresponds to that of compound 504, was nontoxic. The lower biological activity of R595 lipid A than compound 506 may also be explained by the fact that this bacterial product contains compounds of 4'-monophosphate structure.

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