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Immunobiological Activities of Synthetic Lipid A Analogs and Related Compounds as Compared with Those of Bacterial Lipopolysaccharide, Re-Glycolipid, Lipid A, and Muramyl Dipeptide

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Thirteen acylated and phosphorylated derivatives of β-1,6-linked glucosamine disaccharide (lipid A analogs), which were synthesized after the structural model of *Salmonella*-type lipid A, and seven similar derivatives of glucosamine monosaccharide (lipid A-related compounds) were studied for their immunobiological activities. These included mitogenicity and polyclonal B cell activation enhancement of migration of monocytes and polymorphonuclear leukocytes derived from human peripheral blood, stimulation of guinea pig peritoneal macrophages, activation of human complement, and stimulation of serum antibody production and induction of delayed-type hypersensitivity against ovalbumin in guinea pigs. Comparisons were made with lipid A, Re-glycolipid, lipopolysaccharide of natural sources, and a well-known synthetic adjuvant, *N*-acetylmuramyl-L-alanyl-D-isoglutamine. Some of the lipid A analogs were found to manifest the mitogenic, polyclonal B cell-activating macrophage-stimulating, complement-activating, and immunostimulating activities, although the observed activities were generally far less than those of natural products in intensity and efficiency. Other immunobiological effects exhibited by most of the synthetic lipid A analogs were the enhancement of migration of monocytes and polymorphonuclear leukocytes. It is premature to draw definite conclusions on structure-activity relationships, since a few compounds which were active in some assay systems were scarcely active in other assays. However, an indisputable fact was that β-1,6-glucosamine disaccharide 1α,4′-diphosphate, which carries two amide-bound (*R*)-3-hydroxytetradecanoyl and three ester-bound tetradecanoyl residues, and thus has the structure most closely resembling natural lipid A among test compounds in this study, was definitely active in all of the present assay systems. However, its potency was generally much less than natural products. Some of glucosamine monosaccharide derivatives, especially *N*-(*R*)-3-[(*R*)-3-hydroxytetradecanoyloxy]tetradecanoyl glucosamine, also exerted all of the in vitro activities described above. This fact suggests that a glucosamine disaccharide structure may not necessarily be a prerequisite as far as the in vitro immunobiological activities tested are concerned.

Lipopolysaccharide (LPS), one of the main constituents of the outer membrane of gram-negative bacteria, manifests O-antigenic and endotoxic activities and plays an important role in the pathogenesis of gram-negative bacterial infections. A number of studies (8, 22, 29, 35) have clearly demonstrated that most, if not all, immunobiological or immunopharmacological activities of the endotoxic LPS are carried by a lipid A portion of the molecule. Recent chemical analyses (8, 22, 29–31, 35) have revealed that the lipid A moiety of endotoxic LPS of gram-negative bacteria is composed of β-1,6-linked D-glucosamine disaccharides, which carry substituted phosphoryl groups and both ester- and amide-bound fatty acids, especially (*R*)-3-hy-

droxyacyl and (*R*)-3-acyloxyacyl (double acyl) residues. However, there are considerable species variations and significant minor heterogeneity, even within one species, in the chemical structure of "natural" lipid A specimens prepared from LPS. This fact results in the obscurity of the exact chemical structure of the lipid A molecule.

Recently (11a–15; S. Kusumoto et al. *in* L. Anderson and S. M. Unger, ed., *Carbohydrates in Bacterial Lipopolysaccharides: Synthesis and Biomedical Significance*, in press) a series of acylated and phosphorylated derivatives of β-1,6-glucosamine disaccharides were synthesized after the model of *Salmonella*-type lipid A to elucidate the minimum essential structure of the lipid A molecule responsible for its immunobiological activities. Synthetic studies should be very useful for the clarification of the structure-activity relationships of lipid A and related compounds and for the establishment of a standard (reference) specimen of the lipid A to meet the demand of both experimental and clinical studies. Furthermore, approaches along this line may lead us to the creation of novel compounds with immunomodulating activities useful in both clinical and preventive medicine.

In this study, we examined some immunobiological activities of synthetic lipid A analogs and related compounds in comparison with lipid A and LPS preparations from natural sources. *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP), which is a minimal structure responsible for a number of biological response-modifying activities of bacterial cell wall peptidoglycans (1, 5, 20), was also used as another reference.

MATERIALS AND METHODS

Test materials. (i) LPS. LPS prepared by the Boivin method (LPS-B) (4) or the Westphal method (LPS-W) (36) from *Salmonella enteritidis* and *Escherichia coli* were purchased from Difco Laboratories (Detroit, Mich.). An LPS-W specimen prepared in the laboratory of N. Kasai from *Pseudomonas aeruginosa* ATCC 7700 (7) was also used.

(ii) Re-glycolipid. Two reference specimens of Re-glycolipid from *Salmonella minnesota* R595 were used. One (Re-glycolipid-1) was prepared as described previously (6), and the other (Re-glycolipid-2) (18) was a gift of Dennis W. Watson (University of Minnesota, Minneapolis, Minn.) through Kozo Inoue (Osaka University Medical School, Osaka, Japan).

(iii) Lipid A. Lipid A specimens from *S. minnesota* or *P. aeruginosa* were prepared by acid hydrolysis of Re-glycolipid or LPS-W with 1 or 5% acetic acid, respectively (6).

(iv) Synthetic lipid A analogs (acylated and phosphorylated derivatives of β-1,6-glucosamine disaccharide) and similar derivatives of glucosamine. The simple *N*-acyl glucosamine derivatives without phosphate moieties (compounds 308 through 310, see Fig. 1) were prepared from benzyl-2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α- or -β-D-glucopyranoside as follows. After removal of the *N*-acetyl group with Meerwein reagent (12), the free amino group was acylated with tetradecanoyl chloride or with (*R*)- or (*S*)-3-hydroxytetradecanoic acid-dicyclohexylcarbodiimide, respectively (33). Each *N*-acylated compound thus obtained was then treated with concentrated aqueous ammonia in methanol and hydrogenolyzed to give compound 308, 309, or 310. The *N*-[(*R*)-3-[(*R*)-3-hydroxytetradecanoyloxy]tetradecanoyl]glucosamine (compound 313) was prepared from benzyl 2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-D-glucopyranoside in a similar manner by using (*R*)-3-[(*R*)-3-hydroxytetradecanoyloxy]tetradecanoic acid–dicyclohexylcarbodiimide for *N*-acylation. The final deprotection was effected by hydrogenolysis. The structures and purities of all these products were examined by means of elemental analyses and spectroscopic methods.

All other compounds were prepared as already described (11a–15; Kusumoto et al., in press). Figure 1 presents a list of synthetic compounds used in this study, with their chemical structures, the references describing synthetic methods, and their compound numbers.

In some experiments, D-glucosamine hydrochloride (Nakarai Chemicals, Ltd., Kyoto, Japan) was also used as a reference.

(v) MDP. MDP was kindly supplied by Atsuo Inoue (Daiichi Seiyaku, Co., Ltd., Tokyo, Japan).

Determination of mitogenicity. (i) Animals. Male BALB/c and athymic BALB/c nu/nu mice (6 to 8 weeks old) were purchased from commercial farms (Charles River Japan, Osaka, and Clea Japan, Osaka, respectively).

(ii) Lymphocytes. Spleens removed aseptically from the above mice were minced with a cytosieve to gain a splenocyte suspension by the conventional method.

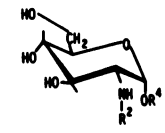
(iii) Test materials. Hydrophilic test materials (compounds 318, 319, and 320 and MDP) were dissolved in RPMI 1640 medium (Nissui Seiyaku, Tokyo). The other, more or less hydrophobic, materials were vigorously sonicated in RPMI 1640 medium with an ultrasonic cleaner (model UT-51; Sharp, Osaka) to get a suspension as homogenous as possible.

(iv) Cell culture. Splenocytes (5×10^6 per ml) were suspended in RPMI 1640 medium which was supplemented with 20% fetal bovine serum (Flow Laboratories Inc., McLean, Va.) and antibiotics (100 U of penicillin G and 100 μg of streptomycin per ml). The cell suspensions (0.1 ml each) in a flat-bottomed polystyrene micro tissue culture tray (cell wells 25860; Corning Glass Works, Corning, N.Y.) were added with test specimens (nothing added to the control) in 0.1 ml of RPMI 1640 medium containing the antibiotics. Cultivation was made at 37°C for 48 h in 5% CO_2–95% air.

(v) Incorporation of [^{3}H]thymidine. [^{3}H]thymidine (2 Ci/mmol; Radiochemical Centre, Amersham, Buckinghamshire, England) in 10 μl of physiological saline was added to each culture 24 h before the harvest of cultured lymphocytes. After further incubation for 24 h, cultured cells were harvested with a semiautomatic multiple-cell harvester (LM 101 LABO MASH; Labo Science Co., Ltd., Tokyo), and the thymidine incorporation into the cells was measured by the conventional liquid scintillation method.

The increase of thymidine uptake by a test specimen was expressed as a stimulation index, a ratio of

Compound number	R^1	R^2	R^3	R^4
301	C_{14}	C_{14}	H	H
302	C_{14}	C_{14}-OH	H	H
307	H	C_{14}-OH	H	H
311	C_{14}-OH	C_{14}-OH	H	H
314	C_{14}-OH	C_{14}-O-(C_{14})	H	H
304	C_{14}	C_{14}	P	H
321	C_{14}	C_{14}-OH	P	H
312	C_{14}-OH	C_{14}-OH	P	H
315	C_{14}-OH	C_{14}-O-(C_{14})	P	H
303	C_{14}	C_{14}	H	P
316	C_{14}	C_{14}-OH	H	P
305	C_{14}	C_{14}	P	P
317	C_{14}	C_{14}-OH	P	P



Compound number	R^1	R^2	R^3	R^4
308		C_{14}-OH		H
309		C_{14}		H
310		(S)-C_{14}-OH		H
313		C_{14}-O-(C_{14}-OH)		H
318		C_{14}-OH		P
319		C_{14}-OH		β-P
320		C_{14}		P

C_{14} : tetradecanoyl
C_{14}-OH : (R)-3-hydroxytetradecanoyl
C_{14}-O-(C_{14}) : (R)-3-tetradecanoyloxytetradecanoyl
C_{14}-O-(C_{14}-OH) : (R)-3-[(R)-3-hydroxytetradecanoyloxy]tetradecanoyl
P : $P-(OH)_2$ (O)

FIG. 1. Compound numbers and structures of lipid A analogs (glucosamine disaccharide derivatives) and related compounds (glucosamine monosaccharide derivatives). The references pertaining to the synthesis of these compounds are as follows: compound 301, reference 12; compound 302, reference 13; compounds 307 and 321, Kusumoto et al., in press; compounds 311, 312, 314, 315, 316, and 317, Inage et al., in press; compounds 303, 304, and 305, reference 14; compounds 318, 319, and 320, reference 15; compounds 308, 309, 310, and 313, this work.

disintegrations per minute of a test culture to disintegrations per minute of the respective control culture. All determinations were carried out in quadruplicate cultures to get the mean ± standard error (SE). Phytohemagglutinin (Wellcome Reagents Ltd., Beckenham, England) was used as a reference mitogen.

Determination of PBA. Polyclonal B cell activation (PBA) was determined as follows.

(i) Lymphocyte culture. Splenocytes were prepared from 8-week-old male BALB/c mice (Charles River) as described above. Cells (6×10^6) were suspended in 1.6 ml of RPMI 1640 medium supplemented with 0.2 ml of fetal bovine serum and the antibiotics (see above). The cell suspensions in a flat-bottomed tissue culture tray (16-mm diameter, 24 wells, Nunclon Delta SI; Nunc, Roskilde, Denmark) were added with test specimens (nothing added to the control) in 0.2 ml of RPMI 1640 medium containing the antibiotics and were cultured at 37°C for 5 days in 5% CO_2–95% air.

(ii) Hemolytic PFC assays. The cell cultures prepared as described above were harvested by centrifuging at $100 \times g$ for 10 min and suspended in 0.1 ml of RPMI medium. To each cell suspension was added 0.01 ml of 40% (vol/vol) sheep erythrocyte suspension in phosphate-buffered saline and 0.01 ml of fresh guinea pig serum (as complement). Portions (0.1 ml) of the above mixture containing 5×10^6 or 5×10^5 splenocytes were packed in a Cunningham chamber (Yazawa Kagaku, Tokyo) and incubated at 37°C for 1 h. The number of antibody-secreting cells (per 5×10^6 splenocytes) was determined by counting plaque-forming cells (PFC). The procedure was performed in triplicate for each dose of each specimen, and the mean PFC number and an SE of the mean were calculated. PBA activity was expressed as a ratio of PFC number in a test culture to that in the respective control culture i.e., as a stimulation index.

Assay for the migration of monocytes and PMNL. (i) Separation of cells. Heparinized blood drawn from healthy adult donors was fractionated with Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden). A fraction which was separated as an interface region and enriched with monocytes (the mean proportion of monocytes to total leukocytes was more than 20%), after being washed twice with phosphate-buffered saline, was suspended in Gey balanced salt solution containing 2% bovine serum albumin (BSA; fraction V; Sigma Chemical Co., St. Louis, Mo.) and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [Sigma]) (Gey-BSA; pH was adjusted to 7.0), to give a cell density of 5×10^6 cells per ml. The sedimented cell fraction after Ficoll-Paque fractionation was treated with a solution which contained 8.29 g of NH_4Cl, 1 g of $KHCO_3$ and 0.372 g of disodium EDTA per 1,000 ml of distilled water (pH 7.4) to lyse erythrocytes. Polymorphonuclear leukocytes (PMNL) which survived this treatment were washed three times with PBS (the mean proportion of PMNL to total leukocytes was more than 80%) and suspended in Gey balanced salt solution (pH 7.2) at 2.5×10^6 cells per ml.

(ii) Test materials. Test compounds were either dissolved or suspended as homogenously as possible by ultrasonication in a mixture consisting of 7 parts Gey-BSA and 5 parts gelatin Veronal buffer with 2 M $MgCl_2$ (Gey-BSA-GVB^{2+}; pH was adjusted to 7.0 for monocytes and 7.2 for PMNL).

(iii) Assay for migration. Assay for the migration of monocytes was performed by use of a multiwell chemotaxis assembly (Neuro Probe, Cabin John, Md.) as described previously (26, 27). The migration of PMNL was determined in a similar manner except that a sheet of 3-μm-pore-size filter paper was used instead of the 5-μm sheet used for monocytes and that cultivation

was made for 60 min instead of the 90 min used for monocytes. The assay for each dose of each specimen was carried out in triplicate. Determination was made of the number (per oil immersion microscopic field) of monocytes or PMNL which migrated to the surface of the membrane sheet adjacent to the lower well containing a test specimen to obtain the mean ± SE. The stimulation of the migration of monocytes or PMNL by test specimens was expressed as a ratio of the mean value in a test to that in the respective control.

In all experiments, two reference specimens were used as positive controls: one was a fresh human serum specimen which was activated by LPS-B of *S. enteritidis* by the conventional method and diluted 1:10 (for monocytes) or 1:2 (for PMNL) with Gey-BSA-GVB^{2+}, and the other was *N*-formylmethionyl-leucylphenylalanine (Sigma) at 10^{-8} M for monocytes and 10^{-6} M for PMNL.

Macrophage activation. Activation of guinea pig macrophages was examined by determination of [^{14}C]glucosamine incorporation according to the method previously described (34) with minor modifications. Briefly, thioglycolate-induced peritoneal macrophages were cultured for 3 days in a multiwell plastic culture tray (24 flat-bottomed wells, ca. 1.7 by 1.6 cm; Linbro Scientific, Inc., subsidiary of Flow Laboratories, Inc., Hamden, Conn.) as a monolayer adhered to a plastic surface. During the final 8 h of the culture, macrophages in each well were pulsed with 0.25 μCi of D-[^{14}C]glucosamine (Radiochemical Centre). The macrophage monolayers were washed three times with Hanks balanced salt solution (Nissui Seiyaku Co.) and then dissolved by the addition of 0.5 ml of Lowry alkaline copper solution. A portion of the dissolved cells was assayed for protein content as an indication of the number of adherent macrophages. Another portion was assayed to determine glucosamine incorporation. The assay was carried out in triplicate or quadruplicate with each dose of each specimen. The stimulation index of glucosamine incorporation by macrophages exposed to test specimens was calculated by the following formula:

$$\frac{\text{dpm of } ^{14}\text{C}/\mu\text{g of protein in a test culture}}{\text{dpm of } ^{14}\text{C}/\mu\text{g of protein in the control culture}}$$

Activation of human complement system. This was examined by a method described in a previous paper (16). Briefly, 0.2-ml portions of fresh, pooled human serum were incubated with test materials dissolved or suspended in 0.2 ml of physiological saline at 37°C for 60 min. The reaction mixtures were cooled in an ice bath to stop the reaction and then centrifuged at 9,000 × *g* for 5 min. The 50% hemolytic complement of the supernatant fluid was determined by the method of Mayer (23) and was compared with that of a control serum treated with saline alone. The blocking effect of both ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA; 10 mM) and $MgCl_2$ (5 mM) added to the reaction mixture was determined to see whether the activation of the complement system by test or reference specimens occurred through the classical or the alternative pathway.

Immunopotentiating (immunoadjuvant) activities in vivo. Groups of five albino guinea pigs were immunized by intra-footpad injection of 100 μg of test materials with 1 mg of ovalbumin as a test antigen, both of which were incorporated in 0.2 ml of a water-in-mineral oil emulsion (Freund incomplete adjuvant; Difco). The induction of delayed-type hypersensitivity (DTH) was determined by a corneal test on the 3rd week after the immunization, and the stimulating effect on antibody production was estimated by the quantitative precipitin reaction with serum specimens obtained on the 4th week. Further details of the assay were reported previously (19, 20).

Statistical analysis. In most of the above assays, the statistical significance of the differences between test and respective control groups were examined by Student's *t* test. Significance was determined at the 5 and 1% levels.

RESULTS

Mitogenic activity. We examined the mitogenic effects of synthetic lipid A analogs and related compounds on splenocytes from athymic BALB/c nu/nu mice, namely B cell effects. Figure 2, showing representative results of those of repeated assays, indicates that among 13 glucosamine disaccharide derivatives, compounds 302, 314, and 316 showed distinct mitogenic activities on murine B cells, and compounds 321 and 317 caused slight, but significant, increases in thymidine uptake at the appropriate doses. Concerning glucosamine monosaccharide derivatives, compound 313 exerted a strong mitogenic effect on murine B lymphocytes. The peak response was obtained at dose of 10 μg (3.3 μg in another experiment, data not shown). Compound 318 gave a slight stimulation at doses of 0.1 to 1.0 μg. Overdoses (100 μg) of some monosaccharide derivatives were toxic to cells. The peak response induced by compound 313 was significantly higher than those induced by either active disaccharide derivatives or MDP, but far less than those induced by natural LPS, Re-glycolipid, and lipid A.

PBA activity on BALB/c mouse splenocytes. Representative experiments (Fig. 3) showed that among 13 test glucosamine disaccharide derivatives, compounds 314, 312, 315, 316, 305, and 317 exhibited a weak, but significant, PBA activity. The activity of compound 315 was comparable to that of MDP, but far less than those of natural lipid A, Re-glycolipid, and LPS. Regarding monosaccharide derivatives, compounds 309, 313, 318, 319, and 320 exhibited distinct PBA effects, stronger than those of the disaccharide derivatives. Glucosamine itself was found to be quite inactive.

Enhancement of the migration of monocytes derived from human peripheral blood. (i) Re-glycolipid and lipid A. A control study with natural LPS and related compounds showed that although none of test LPS preparations exerted any enhancing effects on the migration of monocytes, Re-glycolipid and lipid A specimens caused a distinct enhancement of monocyte mi-

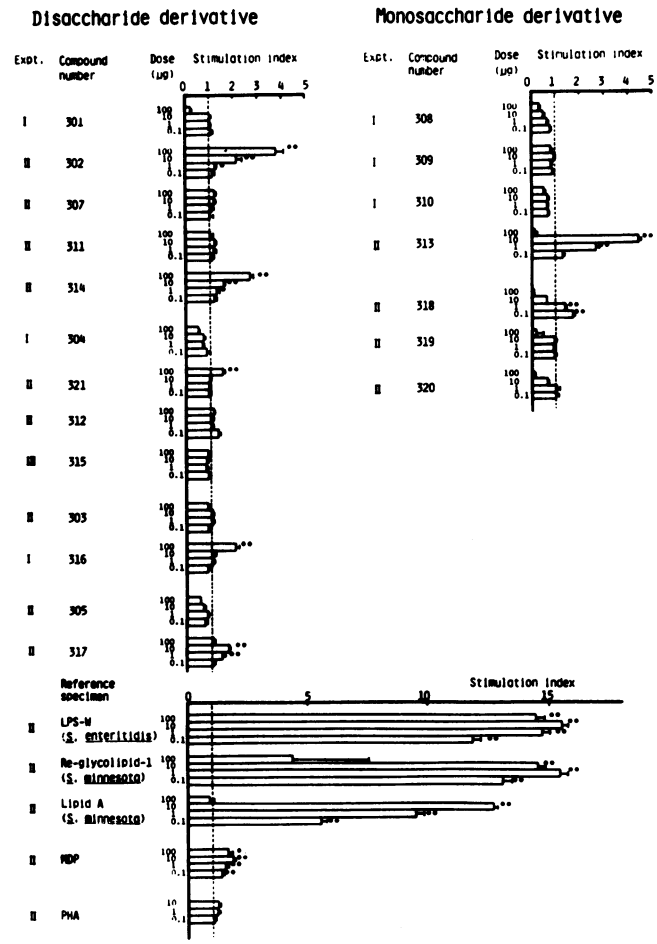


FIG. 2. Mitogenic effect of synthetic lipid A analogs and related compounds on splenocytes from athymic BALB/c nu/nu mice. The cells (5×10^5) were cultured with the indicated doses of test materials (nothing added to control cultures) for 2 days in quadruplicate. The counts in control cultures were: experiment I, 6,267 ± 382 dpm; experiment II, 8,851 ± 242 dpm; experiment III, 9,410 ± 363 dpm. The stimulating activity of a test specimen was expressed as a ratio of test to control values (a stimulation index) ± SE and was examined for statistical significance by Student's *t* test. Symbols: *, significant at the 5% level; **, significant at the 1% level.

gration (Fig. 4). The enhancing effect was comparable to that of MDP in its extent, but the peak response by the former was attained at lower doses than that by the latter, namely, 1 to 10 ng/ml as compared to 100 ng/ml. Table 1 shows the result of a checkerboard analysis which was made to see whether the enhanced migration of monocytes by a lipid A specimen prepared from *S. minnesota* R595 was due to an increased random migration (chemokinesis) or to chemotaxis directed by a concentration gradient of an active agent. It turned out that the enhanced monocyte migration caused by the lipid A was mainly due to chemokinesis, since the intensity of migration enhancement was not significantly affected by the gradient of lipid A concentration between the upper and lower wells.

(ii) Synthetic lipid A analogs and related compounds. The representative assay results summarized in Fig. 5 show that glucosamine disaccharide derivatives, except compounds 301, 302, and 304, exerted moderate to strong migration-enhancing activities. The activities of the most active compounds were comparable in intensity to those of natural lipid A, Re-glycolipid, and MDP. Table 2 shows that the enhanced monocyte migration caused by compound 314 was mainly due to chemokinesis, likewise natural lipid A.

Figure 5 also shows that all of the glucosamine

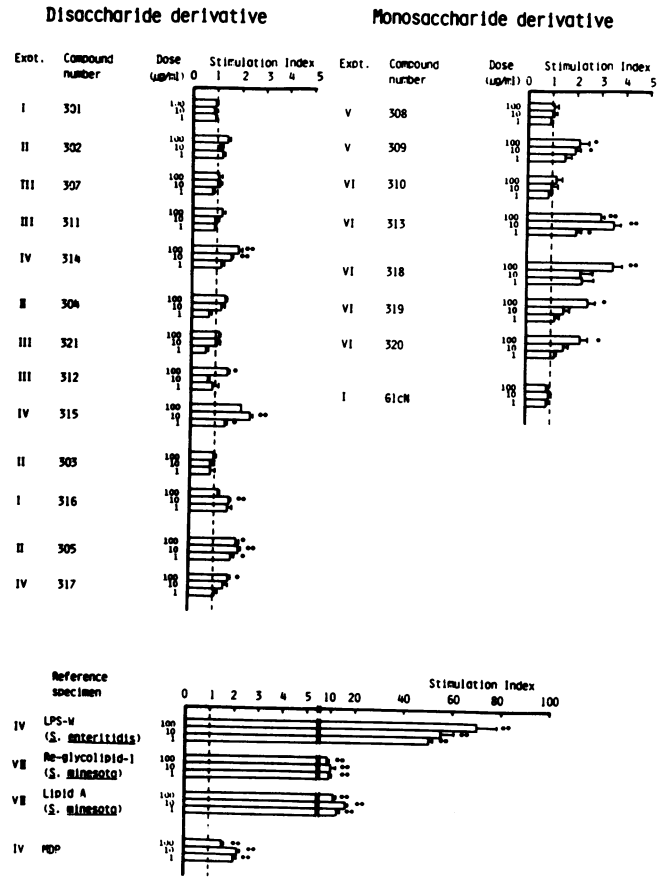


FIG. 3. PBA activities of synthetic lipid A analogs and related compounds on splenocytes from BALB/c mice. The cells (6×10^6) were cultured with the indicated doses of test materials (nothing added to control cultures) for 5 days in triplicate, and the number of direct PFC (per 5×10^6 cells) was determined. The numbers of PFC in control assays were: experiment I, 15.3 ± 0.08; experiment II, 13.0 ± 2.08; experiment III, 19.0 ± 2.08; experiment IV, 20.0 ± 1.58; experiment V, 20.0 ± 1.15; experiment VI, 19.0 ± 3.60; experiment VII, 11.0 ± 1.73. Stimulation index and statistical significance were determined as described in the legend to Fig. 2.

monosaccharide derivatives except compound 320 were more or less active in the enhancement of monocyte migration. The mechanism of migration enhancement, however, does not seem to be identical to those of natural lipid A and synthetic compound 314, since the checkerboard analysis showed that the response of monocytes to concentration gradients of compound 313 was different from responses of monocytes to lipid A and compound 314 (Table 3). The number of monocytes that migrated toward the lower well was almost constant when the concentrations of compound 313 were the same on both sides of the filter, but the monocyte number on the lower surface of the filter increased as the concentration of compound 313 increased in upper wells in the absence of the compound in the lower well. These findings imply that the compound 313 has both chemotactic and chemokinetic effects on human monocytes.

PMNL migration-enhancing activity. Figure 6 shows that all of the natural products, including LPS preparations (except LPS-W of *S. enteritidis*), definitely enhanced the migration of human PMNL. Among the synthetic compounds examined, disaccharide derivatives 311, 314, 303, and 305 and monosaccharide derivatives 309, 310, and 313 caused definite enhancements of PMNL migration (Fig. 7). MDP showed no activity in this test (Fig. 6). The limited amounts available did not permit us to examine the activities of compounds 312, 315, 316, and 317.

Macrophage activation. Figure 8 shows that all of four reference specimens (LPS-W of *P. aeruginosa*, Re-glycolipid of *S. minnesota*, and lipid A from *S. minnesota* and *P. aeruginosa*) significantly stimulated thioglycolate-induced perito-

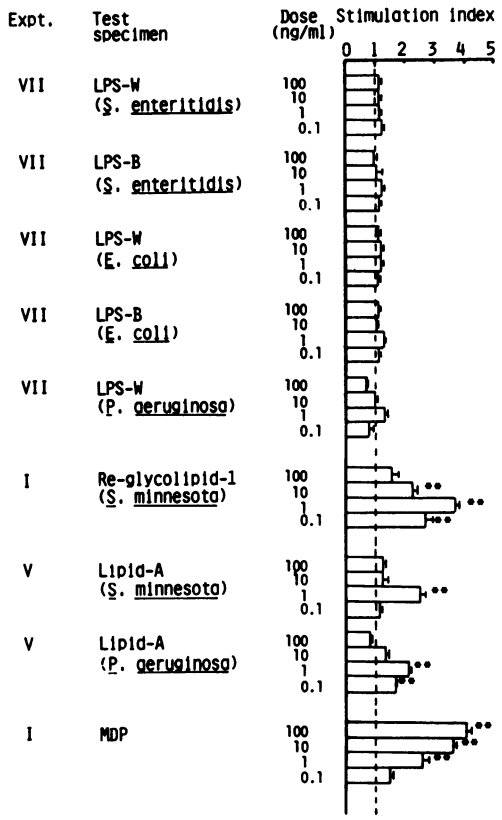


FIG. 4. Monocyte migration in response to various LPS, Re-glycolipid, and lipid A specimens and MDP. Triplicate determinations were carried out on each dose of each specimen. The number of cells per oil immersion field in positive controls and those with no addition were as follows: 7 ± 0 (I), 12 ± 0 (V), and 11 ± 1 (VII) with no addition; 55 ± 2 (I), 78 ± 5 (V), and 71 ± 4 (VII) with 10^{-8} M FMLP; and 66 ± 2 (I), 104 ± 7 (V), and 91 ± 2 (VII) with a 1:10 dilution of LPS-activated serum. Stimulation index and statistical significance were determined as described in the legend to Fig. 2, except that a stimulation index of less than 2 was judged to be insignificant even when the difference between the test and the respective control was found statistically significant, in view of an extremely high sensitivity of the assay method adopted.

neal macrophages to increase the uptake of glucosamine, although there were considerable variations in the extent of stimulation. Among three synthetic compounds which could be submitted to this assay, compounds 317 and 313 exerted a weak, but significant, stimulating activity, whereas compound 316 lacked the activity.

Activation of human serum complement. Figure 9A shows the activation of human complement by LPS, Re-glycolipid, lipid A, and synthetic compounds 316, 317, and 313 (dose-response curves). It can be seen that Re-glycolipid of *S. minnesota* markedly, and LPS-W of *S. enteritidis* and lipid A of *S. minnesota* less markedly but significantly, activated the complement system in fresh, pooled human serum. Among three synthetic compounds examined, compounds 317 and 313 caused a weak, but significant, activation of the human complement, whereas compound 316 was scarcely active. Analysis by the addition of EGTA to block the classical pathway (Fig. 9B) revealed that the activation of human complement by synthetic compound 317 and *S. minnesota* lipid A was markedly suppressed by the blocking effect of EGTA, whereas activation by compound 313, LPS-W of *S. enteritidis*, and Re-glycolipid of *S. minnesota* was only partially blocked by the presence of EGTA in the assay system.

Immunopotentiating activity to induce DTH and to increase serum antibody levels against ovalbumin in guinea pigs. Figure 10 clearly shows that all LPS, Re-glycolipid, and lipid A specimens, when administered to guinea pigs in a form of water-in-mineral oil emulsion, exhibited powerful potentiating effects on the induction of DTH as well as on the production of serum antibody as MDP did. Under the same assay conditions, compounds 302, 307, 311, 314, 312, 315, 316, 305, and 317 significantly increased the formation of anti-ovalbumin precipitating antibody, although their adjuvant effects were weaker than those of natural products used as references (Fig. 11). Among the active compounds, 302, 314, 312, 305, and 317 raised the serum antibody level more than threefold over that of the control animals which received ovalbumin

TABLE 1. Checkerboard analysis of monocyte migration enhanced by natural lipid A prepared from *S. minnesota* R595[a]

Concn (ng/ml) of lipid A in lower well	Monocytes per oil immersion field ± SE at a lipid A upper well concn (ng/ml) of[b]:			
	0	1	5	10
0	**14 ± 2**[c]	27 ± 2	29 ± 4	36 ± 2
1	23 ± 3	**26 ± 3**	28 ± 2	24 ± 0
5	25 ± 2	26 ± 1	**20 ± 1**	28 ± 1
10	29 ± 1	24 ± 0	26 ± 5	**29 ± 2**

[a] Test specimen was added to both wells or either of the lower and upper wells of a multiwell chemotaxis chamber at the indicated concentrations. Monocytes were added to the upper wells.

[b] The monocytes per oil immersion field on the lower surface of a polycarbonate filter were counted in triplicate on 20 microscopic fields to obtain the mean ± SE.

[c] Boldface numbers indicate those values obtained when the concentrations of specimen were the same on both sides of the filter.

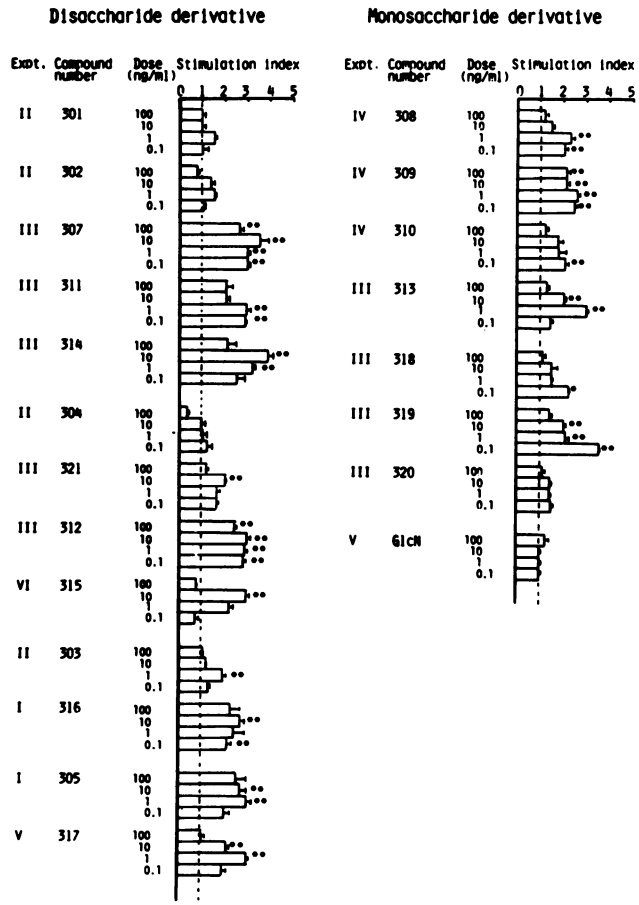


FIG. 5. Monocyte migration in response to synthetic lipid A analogs and related compounds. Determinations were carried out in triplicate on each dose of each specimen. The number of cells per oil immersion field in positive controls and those with no addition were as follows: 7 ± 0 (I), 13 ± 1 (II), 12 ± 2 (III), 11 ± 1 (IV), 12 ± 0 (V), and 12 ± 1 (VI) with no addition; 55 ± 2 (I), 67 ± 2 (II), 68 ± 2 (III), 73 ± 1 (IV), 78 ± 5 (V), and 59 ± 2 (VI) with 10^{-8} M FMLP; and 66 ± 2 (I), 64 ± 1 (II), 92 ± 1 (III), 78 ± 3 (IV), 104 ± 7 (V), and 92 ± 2 (VI) with a 1:10 dilution of LPS-activated serum. Stimulation index and statistical significance were determined as described in the legend to Fig. 4.

alone in water-in-mineral oil emulsion (Fig. 11). With regard to DTH induction, none of test synthetic compounds could induce a strong DTH comparable to those induced by either natural compounds or synthetic MDP. However, the corneal reaction given by animals receiving some compounds (305, 317, and some others) was on the borderline between positive and negative. With regard to monosaccharide derivatives, the assay was made on only compound 313, which was proved to be active by most in vitro assays, although only borderline immunopotentiating effects were noted in vivo (data not shown).

DISCUSSION

In this study we demonstrated that some synthetic lipid A analogs and related compounds (variously acylated and phosphorylated derivatives of β-1,6-glucosamine disaccharide and similar derivatives of glucosamine monosaccharide) exhibit a variety of immunobiological activities inherent in the lipid A portion of LPS (Table 4). The activities detected were obvious, but most of them were far less than those of natural lipid A, Re-glycolipid, or LPS in terms of the intensity and effective dose.

A few groups of Japanese workers and Lüderitz and his colleagues are now studying the immunobiological activities of the same synthetic compounds examined here, from various points of view. The findings have been briefly reported previously (Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., Susono City, Shizuoka, Japan, 7 to 9 November 1982; Jpn. J. Bacteriol., vol. 38, no. 1, a special

TABLE 2. Checkerboard analysis of monocyte migration enhanced by compound 314[a]

Concn (ng/ml) of compound 314 in lower well	Monocytes per oil immersion field ± SE at a compound 314 upper well concn (ng/ml) of[b]:			
	0	0.001	0.01	0.1
0	**19 ± 2**[c]	19 ± 1	45 ± 2	53 ± 2
0.001	27 ± 1	**24 ± 0**	24 ± 1	25 ± 1
0.01	29 ± 1	26 ± 1	**32 ± 1**	27 ± 1
0.1	39 ± 1	25 ± 2	29 ± 1	**38 ± 2**

[a] Test specimen was added to both wells or either of the lower and upper wells of a multiwell chemotaxis chamber at the indicated concentrations. Monocytes were added to the upper wells.

[b] The monocytes per oil immersion field on the lower surface of a polycarbonate filter were counted in triplicate on 20 microscopic fields to obtain the mean ± SE.

[c] Boldface numbers indicate those values obtained when the concentrations of specimen were the same on both sides of the filter.

issue for the 56th Annu. Meet. Jpn. Soc. Bacteriol.).

Among the above studies, Kumazawa's group (Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., p. 20; Jpn. J. Bacteriol. **38**:B-III-8) and Kanagasaki and Yasuda et al. (Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., p. 19; Jpn. J. Bacteriol. **38**:B-III-6) have examined the B cell mitogenic activities of synthetic lipid A analogs. There are some discrepancies between our findings and theirs. For instance, Kumazawa's group and Yasuda's group reported that compound 303 exerted a distinct mitogenic effect, whereas this compound was quite inactive in our assay, and the reverse was the case with compound 302. As a whole, their assay method seems to have been more sensitive for the detection of mitogenic effects than ours. This might be partly due to differences in preparation of the test materials added to splenocyte cultures between their study and ours; we just dispersed test materials in RPMI 1640 medium by ultrasonication, whereas Kumazawa et al. tried to solubilize test specimens by the addition of triethylamine-BSA with reference to reports by Galanos et al. (9, 10), and Kanagasaki's group used test speci-

TABLE 3. Checkerboard analysis of monocyte migration enhanced by compound 313[a]

Concn (ng/ml) of compound 313 in lower well	Monocytes per oil immersion field ± SE at a compound 313 upper well concn (ng/ml) of[b]:			
	0	0.0125	0.025	0.05
0	**18 ± 1**[c]	29 ± 2	32 ± 3	30 ± 1
0.0125	16 ± 1	**19 ± 1**	18 ± 1	26 ± 2
0.025	21 ± 1	23 ± 2	**18 ± 1**	19 ± 2
0.05	26 ± 1	25 ± 1	21 ± 0	**15 ± 1**

[a] Test specimen was added to both wells or either of the lower and upper wells of a multiwell chemotaxis chamber at the indicated concentrations. Monocytes were added to the upper wells.

[b] The monocytes per oil immersion field on the lower surface of a polycarbonate filter were counted in triplicate on 20 microscopic fields to obtain the mean ± SE.

[c] Boldface numbers indicate those values obtained when the concentrations of specimen were the same on both sides of the filter.

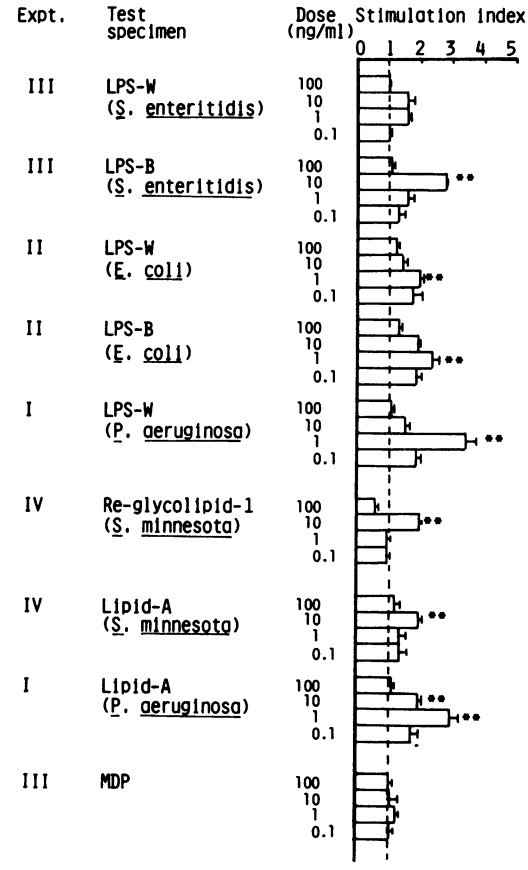


FIG. 6. PMNL migration in response to various LPS, Re-glycolipid, and lipid A preparations and MDP. Determinations were carried out in triplicate on each dose of each specimen. The number of cells per oil immersion field in positive controls and those with no addition were as follows: 12 ± 1 (I), 27 ± 1 (II), 10 ± 1 (III), and 18 ± 1 (IV) with no addition; 124 ± 7 (I), 144 ± 4 (II), 118 ± 5 (III), and 138 ± 6 (IV) with 10^{-6} M FMLP; and 117 ± 2 (I), 227 ± 8 (II), 166 ± 10 (III), and 163 ± 3 (IV) with a 1:2 dilution of LPS-activated serum. Stimulation index and statistical significance were determined as described in the legend to Fig. 4.

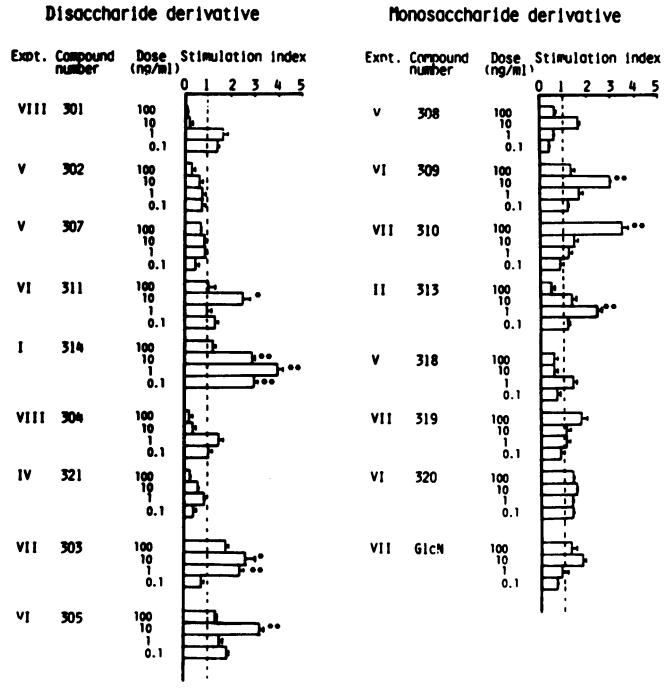


FIG. 7. PMNL migration in response to synthetic lipid A analogs and related compounds. Determinations were carried out in triplicate on each dose of each specimen. The number of cells per oil immersion field in positive controls and those with no addition were as follows: 12 ± 1 (I), 27 ± 1 (II), 18 ± 1 (IV), 11 ± 1 (V), 6 ± 0 (VI), 8 ± 1 (VII), and 12 ± 1 (VIII) with no addition; 124 ± 7 (I), 144 ± 4 (II), 138 ± 6 (IV), 105 ± 8 (V), 64 ± 6 (VI), 64 ± 2 (VII), and 76 ± 3 (VIII) with 10^{-6} M FMLP; and 117 ± 2 (I), 227 ± 8 (II), 163 ± 3 (IV), 129 ± 11 (V), 92 ± 6 (VI), 84 ± 6 (VII), and 155 ± 4 (VIII) with a 1:2 dilution of LPS-activated serum. Stimulation index and statistical significance were determined as described in the legend to Fig. 4.

mens incorporated in multilayered liposomes. It may be added here that some of the disaccharide derivatives were not easy to suspend homogenously in water, and Tanamoto and Lüderitz et al. (Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., p. 18) reported that succinylation of some synthetic lipid A analogs increased their solubility in water and consequently revealed their pyrogenicity, mitogenicity, and activity to inhibit the mitogenicity of lipid A, which had not been detected in original compounds. For example, succinylated compounds 302 and 314 acquired mitogenic activity on murine splenocytes. They also reported that compounds 321, 316, and 317 could exert mitogenicity without succinylation, whereas compounds 304, 312, and 305 lacked mitogenicity at all. Their findings are, as a whole, more compatible with ours than those of Kumazawa's group and Yasuda's group, although no data are available on compound 303 at present. Another finding worthy of note was that a hydroxy double acyl, i.e., 3-(3-hydroxyacyloxy)acyl, derivative of glucosamine (compound 313) showed a distinct B cell mitogenicity, stronger than those of the active disaccharide derivatives.

Concerning PBA activity, too, there are some disagreements between our results and those of Kumazawa's group (Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., p. 20; Jpn. J. Bacteriol. 38:B-III-8); namely, they reported that compound 303 showed a distinct PBA activity but compound 314 did not, and the reverse was the case in our assay. It should be noted that some monosaccharide derivatives (compounds 313 and 318) were definitely active. This and the above findings imply that the glucosamine disaccharide structure of lipid A was not necessarily required for the manifestation of its PBA and B cell mitogenic activities.

In the course of studies on migration-enhancing effects of bacterial cell walls and muramylpeptides on human peripheral monocytes, control experiments showed that lipid A and Reglycolipid enhanced monocyte migration by chemokinesis, although LPS was inactive in this respect (cell walls and MDP enhanced monocyte migration by chemotaxis) (26, 27). The assay was then performed on synthetic lipid A analogs and related compounds and revealed that many test compounds (glucosamine monosaccharide derivatives as well as disaccharide derivatives)

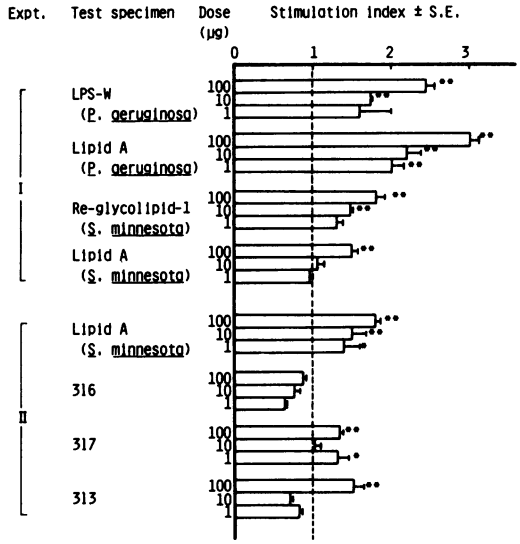


FIG. 8. Stimulation of glucosamine incorporation of peritoneal macrophages from guinea pigs by LPS, Re-glycolipid, lipid A, and synthetic compounds 316, 317, and 313. The cells (10^6) were cultured with indicated doses of test materials (none in controls) for 3 days in triplicate. The counts in control cultures were 199 ± 13.7 dpm (I) and 81 ± 3.5 dpm (II), respectively. Stimulation and statistical significance were determined as described in the legend to Fig. 2.

exhibited the migration-enhancing activity, comparable in intensity to natural lipid A. Although it is not known whether the observed chemokinesis by natural lipid A and synthetic compounds is due to a direct action on monocytes or whether it goes through chemical mediators derived from other blood cells, the above finding may add a new immunobiological activity to the extensive list of activities of lipid A. In this connection, Yoshida et al. (Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., p. 23) observed that some glucosamine disaccharide derivatives (compounds 307, 311, 314, and 312) exhibited a leukocyte migration effect in vivo in mice; namely, activity to decrease the number of nucleated cells in bone marrow 18 h after an intraperitoneal injection of test materials. Compound 307, which enhanced monocyte migration profoundly in our assay system, could exhibit more than threefold the activity of natural lipid A.

There have been a number of studies on the chemotaxigenic effect (an activity to induce chemotactic mediators) of LPS on PMNL in the presence of fresh serum. There are no reports on the chemotactic activity of LPS on PMNL, except a recent study of Adamu and Sperry (2) who examined the chemotactic effects of *Bacteroides* culture filtrate and outer membrane on rabbit PMNL. They showed, in contrast to a negative result reported by Sveen (32) with an LPS-W of *Veillonella alcalescens*, that an LPS-W preparation of *Bacteroides fragilis* which was used as a reference exerted a migration-enhancing activity on rabbit PMNL without the addition of serum as a source of mediators. We

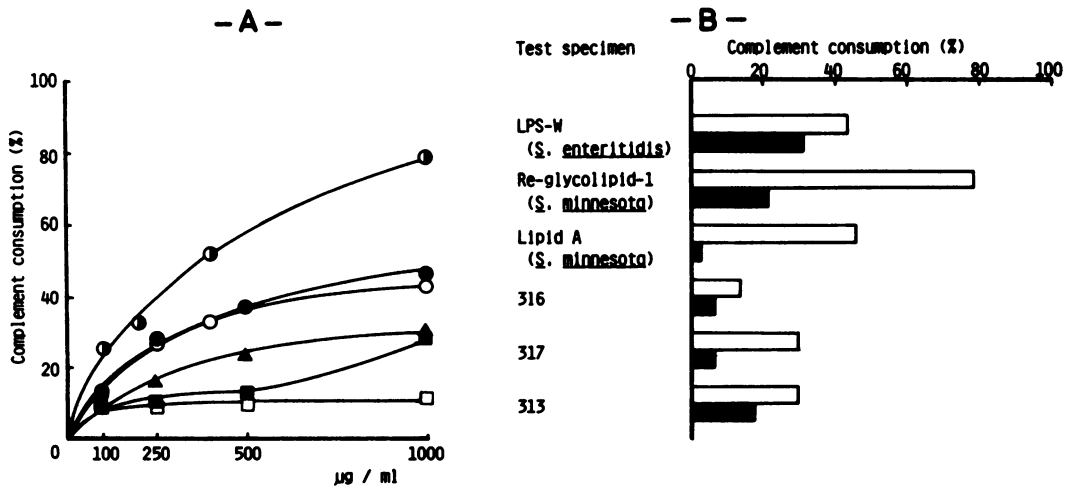


FIG. 9. Activation of human complement system by LPS, Re-glycolipid, lipid A, and synthetic compounds 316, 317 and 313. Dose-response curves (A) and the blocking effects of the classical pathway by addition of EGTA (B). (A) Symbols: ○, LPS-W of *S. enteritidis*; ◑, Re-glycolipid of *S. minnesota*, ●, lipid A of *S. minnesota*; □, compound 316; ▲, compound 317; ■, compound 313. (B) Open and closed columns represent the complement consumption (decrease of 50% hemolytic complement in the absence and the presence of EGTA (10 mM) and $MgCl_2$ (5 mM), respectively. Test specimens were added to pooled, fresh human serum at a concentration of 1 mg/ml of reaction mixture.

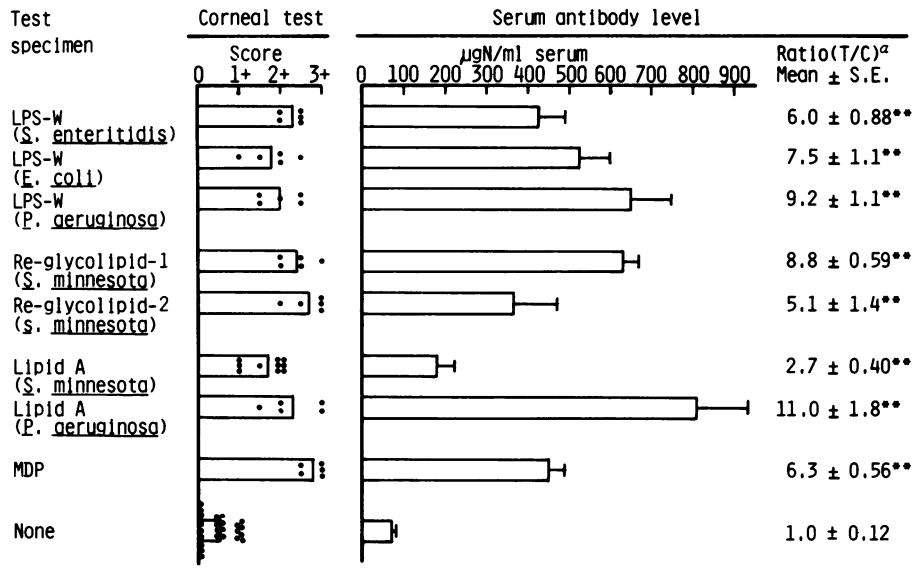


FIG. 10. Adjuvant activity of LPS, Re-glycolipid, and natural lipid A to induce DTH and to enhance antibody production in guinea pigs. Groups of five albino guinea pigs were immunized by intra-footpad injection of a water-in-mineral oil emulsion containing ovalbumin (1 mg) and test specimen (100 μg). The induction of DTH was examined by corneal test, and the serum anti-ovalbumin antibody level was determined by the quantitative precipitin method 3 and 4 weeks after the immunization. With corneal reaction, each dot represents score (arbitrarily graded with a maximum of 3+) of an individual animal and each column shows the mean score. Serum antibody level was expressed as micrograms of antibody nitrogen per milliliter of serum, and the ratio (*a*) of antibody content in a test group to that in a control group was calculated (mean ± SE). Stimulation index and statistical significance were determined as described in the legend to Fig. 2.

demonstrated that the migration of human peripheral PMNL was significantly stimulated by LPS, Re-glycolipid, and lipid A at much smaller doses (1 to 100 ng/ml) (Fig. 6) than the effective dose described above. Furthermore, we found that some of the synthetic compounds showed a similar migration-enhancing effect on PMNL (Fig. 7), although we could not test the activity of other compounds because of their limited amount.

There are many studies showing that LPS activates macrophages in various ways (25). Among them, Wilton et al. (37) reported that LPS increased the glucosamine uptake by guinea pig peritoneal macrophages in the presence of B lymphocytes. We first confirmed that this activity of LPS was carried by its lipid A portion, like other immunobiological activities, and then examined possible similar activities for two disaccharide derivatives (compounds 316 and 317) and one monosaccharide derivative (compound 313). Although compound 316 was inactive, compounds 317 and 313 gave distinct positive results. It is not clear at present whether the observed activity of these specimens was due to indirect effects via lymphocytes as reported by Wilton et al. (37) with LPS or to direct effects on macrophages. In this connection, Kumazawa et

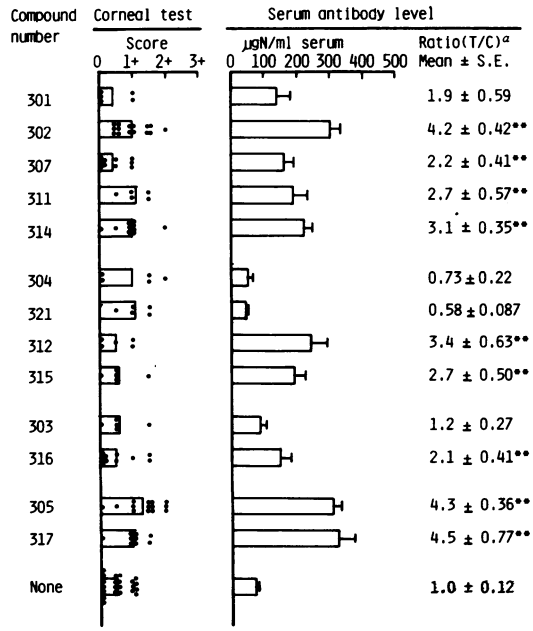


FIG. 11. Adjuvant activities of synthetic lipid A analogs and related compounds to induce DTH and to enhance antibody production in guinea pigs. See the legend to Fig. 10.

TABLE 4. Summary of immuobiological activities of synthetic lipid A analogs (glucosamine disaccharide derivatives) and related compounds (glucosamine monosaccharide derivatives)[a]

Test specimen	B-cell mitogenicity	Polyclonal effects	Enhanced migration		Macrophage stimulation	Complement activation	Adjuvanticity	
			Monocytes	PMNL			DTH	Ab
Disaccharides								
301	−	−	−	−			−	−
302	+	−	−	−			±	2+
307	−	−	2+	−			−	±
311	−	−	+	+			±	±
314	+	+	2+	2+			±	±
304	−	−	−	−			±	−
321	±	−	+	−			±	−
312	−	+	2+				−	+
315	−	2+	2+				−	±
303	−	−	+	+			−	−
316	+	+	2+		−	±	−	±
305	−	2+	2+	2+			±	2+
317	±	+	2+		+	+	±	2+
Monosaccharides								
308	−	−	+	−				
309	−	+	+	2+				
310	−	−	+	2+				
313	2+	2+	2+	+	+	+	−	±
318	±	2+	+	−				
319	−	+	+	−				
320	−	+	−	−				
References								
LPS[b]	3+	3+	−	+	2+	2+	2+	3+
Re-glycolipid[b]	3+	3+	2+	+	2+	2+	2+	3+
Lipid A[b]	3+	3+	2+	2+	2+	2+	2+	3+
MDP	+	+	2+	−	+[c]	−[d]	3+	3+

[a] The activities of the test materials were graded from − to 3+ on the basis of the intensity and statistical significance of the stimulation, the effective dose, and the reproducibility in repeated assays.

[b] Determined by judgment of the data obtained with test preparations of the same category but from different sources.

[c] From reference 34.

[d] From reference 17.

al. (Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., p. 20; Jpn. J. Bacteriol. **38**:B-III-8) found that although natural lipid A induced cytostatic macrophages, none of their synthetic lipid A analogs exhibited such activity.

Morrison and Kline (24) demonstrated that LPS activated both classical and alternative pathways of the complement system and that the lipid A region of the LPS was responsible for the activation of classical pathway, but by a mechanism independent of the antigen-antibody reaction. After confirmation of their finding by demonstrating that lipid A of *S. minnesota* activated the human complement system almost exclusively via the classical pathway, whereas LPS of *S. enteritidis* and Re-glycolipid of *S. minnesota* did so through both pathways, we submitted three synthetic compounds to the assay. Among them, compound 317 was found to activate human complement system mainly via the classical pathway, like natural lipid A. Another disaccharide derivative, compound 316, was scarcely active, and interestingly enough, a monosaccharide derivative, compound 313, caused a weak but significant complement-activating activity via both the classical and alternative pathways. There is a good agreement with an observation of Tanamoto and Lüderitz et al. (Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., p. 18) who noted the activation of human complement by compound 317, but not by compound 316.

A number of studies have demonstrated by both in vitro and in vivo assays that endotoxic LPS works as a potent adjuvant in stimulation of antibody production. However, only very limited information (11, 21, 28) is available on the activity of LPS to induce DTH, one of the protypes of cell-mediated immunity. In the present study, we showed that all three LPS preparations were highly active in the ability to induce DTH (positive corneal reaction) as well as to elevate serum antibody level, and we further demonstrated that these adjuvant activities

could be located in the lipid A region, in a way similar to most other immunobiological activities of LPS. Concerning the antibody production, some of the disaccharide derivatives, particularly compounds 302, 305, and 317, when administered to guinea pigs as a water-in-mineral oil emulsion with ovalbumin, obviously increased the production of anti-ovalbumin precipitating antibody. Our result is partly inconsistent with those of Yasuda et al. (38; Jpn. J. Bacteriol. **38**:B-III-6) and Kanagasaki et al. (Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., p. 19) who examined the adjuvant activity of glucosamine disaccharide derivatives for increasing the number of IgM PFC in mouse spleens which received an intraperitoneal injection of liposomes containing test specimens and trinitrophenylaminocaproylglycerophosphoethanolamine as a test antigen. We noted a definite activity for compound 302 and no activity for compound 303, whereas the reverse was the case in their studies. The discrepancies may derive from differences in experimental conditions, such as test antigens, animal species, vehicles for administration and assay methods. It may be added here that a hydroxy double acyl derivative of glucosamine (compound 313) which showed a number of in vitro immunobiological activities sometimes more distinct than those of disaccharide derivatives, was proved to be only marginally active in the immunoadjuvant assays described in the preceding paragraphs.

Studies have been made in several laboratories of immunobiological activities other than those described here with the same synthetic lipid A analogs. Kojima et al. (Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., p. 21) and Matsuura et al. (Jpn. J. Bacteriol. **38**:B-III-9) reported the interferon-inducing activity of compounds 312, 315, 303, and 316. They noted a very weak activation of the proclotting enzyme of the horseshoe crab with only phosphorylated lipid A analogs. Tanamoto and Lüderitz et al. (Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., p. 18) observed pyrogenic and lethal activities for compounds 316 and 317 and slight pyrogenicity for compound 321, whereas Matsuura et al. (Jpn. J. Bacteriol. **38**:B-III-9) reported nonpyrogenicity for all of the synthetic lipid A analogs examined. Kasai et al. (Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., p. 22) observed that disaccharide derivatives; compounds 302, 314, 315 and 317; and a monosaccharide derivative, compound 313, have a weak activity to prepare the rabbit skin to Shwartzman's reaction provoked by *S. minnesota* Re-glycolipid. Ogawa et al. (personal communication) noted the suppressive activity of compounds 316 and 317 against Meth-A growth in BALB/c mice, comparable in potency to *Nocardia* cell wall skeleton, a well-known antitumor bacterial product (3).

In view of available information, which is limited at present and somewhat inconsistent, it would not be advisable to draw definite conclusions about structure-activity relationships among synthetic lipid A analogs. Surveying the findings described in this report, however, it is tempting to suppose that 3-hydroxylation of a tetradecanoyl residue on the R^2 position (see Fig. 1) tends to increase the activities, as illustrated by the fact that the activities of compounds 302, 321, 316, and 317 are, as a whole, more obvious than those of compounds 301, 304, 303, and 305, respectively. This does not seem to be true with the acyl groups at the R^1 position. For example, compounds 305 and 317 were the most active compounds in a majority of the assays, although they are devoid of the hydroxyl groups on tetradecanoyl residues. Moreover, the introduction of a double acyl residue at the R^2 position also tends to increase various activities; namely, compounds 314 and 315 were, as a whole, more active than compounds 311 and 312, respectively. Another general tendency is that the presence of phosphoryl groups at the R^3 or R^4 positions more or less increases various biological activities. Thus, compound 317, a β-1,6-linked glucosamine disaccharide which carries an (*R*)-3-hydroxytetradecanoyl group at the R^2 position, tetradecanoyl residues at the R^1 position, and phosphoryl groups at the R^3 and R^4 positions, proved to be the most active among glucosamine disaccharide derivatives. This compound was shown to be active in almost all assays carried out by the above-mentioned Japanese and German research groups: pyrogenicity, lethal toxicity, immunopotentiating activities, the activities to suppress tumor growth and to prepare the Shwartzman reaction, modification of hepatic cytochrome P-450 activity in vivo, B cell mitogenicity, PBA activity, macrophage activation, migration enhancement of monocytes, antigenicity, and complement activation in vitro. The activities of compound 317 were comparable to those of MDP in in vitro assays, but were far less than those of natural lipid A in both intensity and efficacy on a weight basis. The possibility that the immunobiological activities of compound 317 are due to LPS as contaminants can be excluded by the fact that some lipid A analogs (for example compounds 301 and 304) which were synthesized in the same laboratory by similar methods were quite inactive.

A remarkable finding revealed by the present study is that some acyl derivatives of glucosamine, particularly compound 313, *N*-[(*R*)-3-[(*R*)-3-hydroxytetradecanoyloxy]tetradecanoyl] gluco-

samine, exhibited distinct immunobiological activities in all of the in vitro assay and borderline activity in adjuvancy in vivo, although this compound was found to be quite inactive in other in vivo assays (pyrogenicity and lethality [Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., p. 18]). This finding would permit us the tentative statement that lipid A does not necessarily need a glucosamine disaccharide structure as a backbone to manifest some of its immunobiological activities in vitro. However, the possibility still remains that the mode of action of compound 313 is entirely different from that of natural lipid A or the active glucosamine disaccharide derivatives as shown in complement-activating activity (Fig. 10) and monocyte migration-enhancing activities (Tables 1, 2, and 3) of the specimen. Kumazawa et al. (Int. Symp. Chem. Biol. Aspects Bacterial Endotoxin Relat. Prod., p. 20; Jpn. J. Bacteriol. **38**:B-III-8) reported that compound 314, unlike other mitogenic synthetic compounds, showed a significant mitogenicity on splenocytes from C3H/HeJ (a nonresponder to LPS) as well as from C3H/HeN mice. In view of these findings, the above possibility should be verified by further study with low responders and nonresponders to LPS or lipid A before forming a hasty conclusion.

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