INFECTION AND IMMUNITY, Jan. 1988, p. 149–155
0019-9567/88/010149-07$02.00/0

Vol. 56, No. 1

Importance of Fatty Acid Substituents of Chemically Synthesized Lipid A-Subunit Analogs in the Expression of Immunopharmacological Activity

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Received 8 July 1987/Accepted 28 September 1987

The immunopharmacological activities of chemically synthesized lipid A-subunit analogs, 4-*O*-phosphono-D-glucosamine derivatives carrying different *N*- and 3-*O*-linked acyl groups, were investigated. None of the synthetic compounds tested exhibited any detectable pyrogenicity at a dose of 10 μg/kg. Weaker lethal toxicity in galactosamine-sensitized mice was detected at 1 μg per mouse for all the synthetic compounds except GLA-58. Among (RS) stereoisomers of 4-*O*-phosphono-D-glucosamine derivatives carrying a 3-*O*-tetradecanoyl (C_{14}) group with different *N*-linked acyloxyacyl groups, i.e., 3-dodecanoyloxytetradecanoyl [C_{14}-O-(C_{12})], 3-tetradecanoyloxytetradecanoyl [C_{14}-O-(C_{14})], and 3-hexadecanoyloxytetradecanoyl [C_{14}-O-(C_{16})] groups (termed GLA-57, GLA-27, and GLA-58, respectively), GLA-27 exhibited significant colony-stimulating factor-inducing and tumor necrosis factor-inducing activities, mitogenicity, polyclonal B-cell activation activity, macrophage activation, and adjuvanticity. The activities of GLA-57, which had an *N*-linked C_{14}-O-(C_{12}) group, were equivalent to or somewhat weaker than those of GLA-27 with a C_{14}-O-(C_{14}) group. Significant immunopharmacological activities were not observed for GLA-58, carrying a C_{14}-O-(C_{16}) group bound to the amino group. GLA-59, carrying 3-*O*-linked 3-hydroxytetradecanoyl (C_{14}OH) and *N*-linked C_{14}-O-(C_{14}) groups, showed much higher activities than GLA-27. GLA-60, a compound which possesses the same fatty acid substituents as GLA-59 but with reversed binding sites, showed the strongest B-cell activation and adjuvant activities among the synthetic compounds. Among stereoisomers of GLA-59 and GLA-60 composed of fatty acid substituents with the (RR) and (SS) configuration, compounds with the (RR) configuration elicited stronger activities than the (SS) stereoisomers. The importance of fatty acid substituents, including stereospecificity for the expression of immunopharmacological activities of 4-*O*-phosphono-D-glucosamine derivatives, was demonstrated.

Recently, many efforts have been focused on the development of lipid A-subunit analogs with low pyrogenicity which possess beneficial biological activities capable of enhancing host defense mechanisms (8–11, 14–16, 19–21). GLA-27, a glucosamine derivative with 4-*O*-phosphoryl, 3-*O*-C_{14}, and *N*-C_{14}-O-(C_{14}) groups, exhibited significant biological activities without pyrogenicity (8–11, 14–16). In previous reports (10, 15, 16), we indicated the importance of fatty acid substituents in synthetic compounds for the expression of biological activities. In addition, significant differences in the biological activities between *Escherichia coli* and *Salmonella*-type lipid A's have been demonstrated by using chemically synthesized lipid A analogs (2, 3, 7). Difference in the chemical structure of both lipid A's is based on the C_{14}OH and C_{14}-O-(C_{16}) groups linked to the amino group in the reducing sugar part (GlcN-I), respectively.

To clarify the relationship between chemical structure and biological activities of lipid A-subunit analogs with special reference to fatty acid substituents, several 4-*O*-phosphono-D-glucosamines carrying different acyl groups were synthesized chemically (4–6). In the synthetic process, we obtained compounds with different stereospecificities, which are attributable to the asymmetric carbon at the C3 position of C_{14}OH, though the configuration of fatty acid substituents of natural lipid A's is of the (R) form. We previously demonstrated the dissociation of biological activities between the (R) and (S) configurations of GLA-27 and GLA-40 (1-deoxy GLA-27) (8). The present investigation was undertaken to determine the biological activities of GLA-59 and GLA-60 with (RR) and (SS) configurations based on the asymmetric carbon in both C_{14}OH groups linked to the amino and hydroxy groups at C2 and C3 of the GlcN backbone.

In this study, both the fatty acid substituents and the stereospecificity of lipid A-subunit analogs were found to be important for the expression of immunopharmacological activities.

MATERIALS AND METHODS

Synthetic compounds and control lipid A. Lipid A-subunit analogs were synthesized chemically (4–6) and purified by high-performance liquid chromatography. The purity of synthetic compounds was more than 99%. The chemical structure of the compounds is given in Fig. 1. GLA-27, GLA-57, and GLA-58 had the (RS) configuration, on the basis of an asymmetric carbon at the C3 position of an *N*-linked C_{14}OH group. Both GLA-59 and GLA-60 had not only (RS) but also (RR) and (SS) stereoisomers, as judged by two asymmetric carbons at the C3 position of C_{14}OH and C_{14}-O-(C_{14}) groups.

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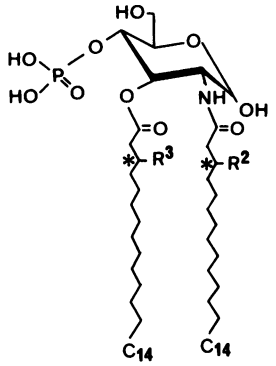

Samples tested	Substituents	
	R^3	R^2
GLA-57	H	C_{12}
GLA-27	H	C_{14}
GLA-58	H	C_{16}
GLA-59	OH	C_{14}
GLA-60	C_{14}	OH

C_{12}: Dodecanoyl C_{14}: Tetradecanoyl

C_{16}: Hexadecanoyl

FIG. 1. Chemical structures of chemically synthesized lipid A-subunit analogs with stereospecificity. *, Asymmetric carbon.

Natural lipid A prepared from *E. coli* F-515 (Re) was kindly donated by O. Lüderitz and C. Galanos (Max-Planck-Institut für Immunbiologie, Freiburg, Federal Republic of Germany) and was used as a control. The synthetic compounds and control natural lipid A were easily solubilized in pyrogen-free water by adding triethylamine complexed with bovine serum albumin (BSA) and were then used for testing biological activities.

Preparation of cells. Spleen cells were prepared from female 10-week-old C3H/He (Shizuoka Animal Center, Hamamatsu, Japan) and C3H/HeJ (The Jackson Laboratory, Bar Harbor, Maine) mice as described previously (11). Macrophages were obtained as adherent cells from the peritoneal cavity of female 10-week-old (BALB/c × DBA/2)F_1 (CDF_1) mice (Shizuoka Animal Center) which had been administered intraperitoneally (i.p.) 20 μg of test samples 4 days earlier as described previously (8). Bone marrow cells were removed from 8- to 10-week-old C57BL/6 mice and incubated for 1 h at a cell density of 5×10^5/ml to remove adherent cells. Resultant nonadherent cells were used as bone marrow cells for assay of colony-stimulating factor (CSF).

Determination of CSF-inducing activity. CSF-inducing activity was determined by a slight modification of the method of Apte et al. (1). Briefly, 10-μg test samples were injected intravenously (i.v.) into 8-week-old ddY mice (Shizuoka Animal Center). After 6 h, sera were separated from five mice per group and pooled to determine CSF activity. The indicated volume of test sera was mixed with 0.1 ml of bone marrow cells (5×10^4), 0.15 ml of fetal calf serum, and 0.25 ml of 1.2% agar (0.3% in final concentration). The mixture was brought to 1 ml with McCoy medium in 35-mm petri dishes and incubated at 37°C for 7 days in a humidified atmosphere of 5% CO_2–95% air. A cell aggregate composed of more than 50 granulocytes and macrophages was counted as one colony.

Determination of TNF-inducing activity. Tumor necrosis factor (TNF)-inducing activity was assessed by the method described previously (3). Briefly, 10-μg test samples were administered i.v. into *Propionibacterium acnes*-primed ICR mice (Charles River Japan Inc., Atsugi, Kanagawa, Japan). Sera were separated from blood 1.5 h after administration and treated at 56°C for 30 min before use. The TNF activity in the sera was determined by measuring the percent inhibition of tritiated thymidine ([^{3}H]dThd) uptake into L929 cells.

Determination of mitogenic activity. Mitogenic activity was assessed by determining [^{3}H]dThd uptake into C3H/He and C3H/HeJ spleen cells which were incubated in triplicate at 37°C for 48 h with or without test samples (11).

Determination of PBA activity. Polyclonal B-cell activation (PBA) activity was assessed by counting as hemolytic plaques antibody-secreting cells which developed by polyclonal activation (11). C3H/He spleen cells were stimulated with 10-μg test samples at 37°C for 48 h. The number of plaque-forming cells elicited against trinitrophenylated horse erythrocytes is represented as the arithmetic mean ± standard deviation of triplicate cultures.

Determination of phagocytic activity. Phagocytic activity was assessed by measuring the radioactivity of ^{51}Cr-labeled, antibody-sensitized sheep erythrocytes which were phagocytosed by peritoneal macrophages stimulated in vivo as described previously (8).

Determination of cellular lysosomal enzyme activity. Cellular lysosomal enzyme (acid phosphatase) activity of peritoneal macrophages was measured by the method described previously (8). Peritoneal macrophages stimulated in vivo were lysed in 96-well microplates with 0.1% Triton X-100. Volumes of 20 μl of 0.2 M acetate buffer (pH 5.0) and 20 μl of 24 mM *p*-nitrophenyl phosphate were added to the resultant macrophage lysates and incubated at 37°C for 30 min. Then 200 μl of 0.2 M sodium carbonate was added to the reaction mixture, and the A_{405} was estimated. Enzyme activity was calculated from the standard curve using acid phosphatase from potato.

Determination of cytostasis-inducing activity. Cytostasis-inducing activity was tested according to the method described previously (8). The activity of peritoneal macrophages stimulated in vivo was assessed by measuring the growth inhibitory action against target EL-4 lymphoma cells, determined as the percent inhibition of [^{3}H]dThd uptake into EL-4 cells.

Determination of adjuvant activity. Adjuvant activity was assessed by measuring anti-BSA immunoglobulin G (IgG) antibody response in the serum of mice which had been immunized i.p. with 10 μg of BSA and 10 μg of test sample 25 days earlier. Anti-BSA IgG antibody titers were determined by an enzyme-linked immunosorbent assay using alkaline phosphatase-conjugated goat anti-mouse IgG serum (1:1,000 dilution, no. 60122; Zymed Laboratories, Inc., San Francisco, Calif.), *p*-nitrophenyl phosphate (1 mg/ml) as a substrate, and BSA-coated 96-well microplates. The intensity of reaction to the sera diluted at 1:1,000 was read as the optical density at 405 nm after incubation for 30 min. Values were calculated by multiplying the optical density value at 405 nm by the serum dilution.

Determination of pyrogenicity, local Shwartzman reaction, and lethal toxicity. Pyrogenicity, local Shwartzman reaction, and lethal toxicity were determined by methods described previously (3). Fever response was recorded after i.v. injection of test samples into 2.5-kg Japanese white rabbits

TABLE 1. Endotoxic activities of chemically synthesized lipid A-subunit analog with the (RS) configuration

Sample tested[a]	Pyrogenicity MED (μg/kg)[b]	Local Shwartzman reaction MPD (μg)[c]	Lethal toxicity LD_{50} (μg)[d]
GLA-57	>10	>50	0.24
GLA-27	>10	>50	0.53
GLA-58	>10	>50	>10
GLA-59	>10	>50	0.56
GLA-60	>10	>50	0.75
Lipid A	0.001	1.25	0.0017

[a] Samples were solubilized with triethylamine and complexed with BSA.
[b] Minimum effective dose (MED) causing pyrogenicity was determined by using three Japanese white rabbits per each dose of the sample.
[c] Minimum preparatory dose (MPD) causing a local Shwartzman reaction was determined by intensity of cutaneous reaction when rabbits were given i.v. a challenge injection of 20 μg of natural lipid A per kg 5 h previously.
[d] Lethal toxicity, expressed as 50% lethal dose (LD_{50}), was calculated by the method of Karber using galactosamine-sensitized mice.

(Gokita Breeding Service, Tokyo, Japan). Three rabbits were used for each dose. The local Shwartzman reaction was tested as follows. Eighteen hours after preparatory intradermal injection with test samples in 0.1 ml of water, a challenge injection of natural lipid A (20 μg/kg) was made i.v. The intensity of reactions was read 5 h after the challenge injection. Lethal toxicity was determined 24 h after i.v. administration of test samples to galactosamine-sensitized mice, as follows: test samples in 0.2 ml of water were injected into male 10-week-old C57BL/6 mice (Shizuoka Animal Center) immediately after i.p. administration of 10 mg of D-galactosamine in 0.5 ml of phosphate-buffered saline.

RESULTS

Pyrogenicity, local Shwartzman reaction, and lethal toxicity. Natural lipid A exhibited remarkable pyrogenicity at a dose of 0.001 μg/kg in Japanese white rabbits (Table 1). None of the synthetic compounds showed any detectable activity at a dose of 10 μg/kg. Whereas natural lipid A induced local Shwartzman reaction at a dose of 1.25 μg, the minimum preparatory dose of all the synthetic compounds for this reaction was more than 50 μg. Weak lethal toxicity in galactosamine-sensitized mice was detected for all compounds except GLA-58 at a dose of 1 μg per mouse.

CSF-inducing activity. Remarkable CSF production was observed in the blood of mice injected i.v. with 10 μg of natural lipid A 6 h previously (Fig. 2). Among compounds with the (RS) stereospecificity, GLA-27, GLA-59, and GLA-60 showed significant CSF-inducing activity, though the activity was weaker than that of natural lipid A. The activity of GLA-57 with an *N*-linked C_{14}-O-(C_{12}) group was weaker than that of GLA-27 with the C_{14}-O-(C_{14}) group. GLA-58, with the C_{14}-O-(C_{16}) group, did not show significant activity.

TNF-inducing activity. Remarkable TNF activity was observed in the blood of *P. acnes*-primed mice which were injected i.v. with natural lipid A (Fig. 3A). All of the synthetic compounds except GLA-58, which had the (RS) configuration, exhibited similarly significant activity, though the activity was weaker than that of lipid A. Lesser activity was seen with GLA-58. Among stereoisomers of GLA-59 and GLA-60 with the (RR) and (SS) configuration at the asymmetric carbon of the C_{14}OH and C_{14}-O-(C_{14}) groups, compounds with the (RR) configuration showed stronger TNF-inducing activity than compounds with the (SS) configuration (Fig. 3B).

Mitogenic activity. Among stereoisomers with the (RS) configuration (Fig. 4A), GLA-27 exhibited significant mitogenic activity, as reported previously (9–11). Weaker mitogenic activity than that of GLA-27 was seen with GLA-57. GLA-60 and GLA-59 showed much higher mitogenic activity than GLA-27. No detectable mitogenic activity was shown by GLA-58. Both GLA-59 and GLA-60 with the (RR) configuration showed much stronger activity than compounds with the (SS) configuration, though significant mitogenic activity was observed in the (SS) stereoisomers of GLA-59 and GLA-60 (Fig. 4B). None of the analogs tested stimulated C3H/HeJ spleen cells with appreciable activity.

PBA activity. Among compounds with the (RS) configuration, the PBA activity of GLA-27 was significant as compared with that of the control and almost equivalent to that of GLA-57 (Table 2). GLA-59 and GLA-60 exhibited somewhat stronger PBA activity than GLA-27. GLA-58 (RS) did not show any detectable PBA activity. Among stereoisomers of GLA-59 and GLA-60, compounds with the (RR) configuration indicated much stronger PBA activity than stereoisomers with the (SS) configuration.

Macrophage activation. Macrophage activation with respect to peritoneal cell accumulation, increment of phagocytosis of ^{51}Cr-labeled, antibody-sensitized sheep erythrocytes, augmented level of cellular lysosomal enzyme (acid phosphatase), and induction of cytostasis is summarized in Table 3. Natural lipid A induced remarkable macrophage activation. Among synthetic compounds with the (RS) configuration, stronger macrophage activation was induced by i.p. administration of either 20 μg of GLA-27 or 20 μg of GLA-60. The activity of GLA-57 and GLA-59 was significant compared with the control, whereas GLA-58 did not induce appreciable macrophage activation. Among stereoisomers of GLA-59 and GLA-60, compounds with the (RR) configuration showed stronger activities than those with the (SS) configuration.

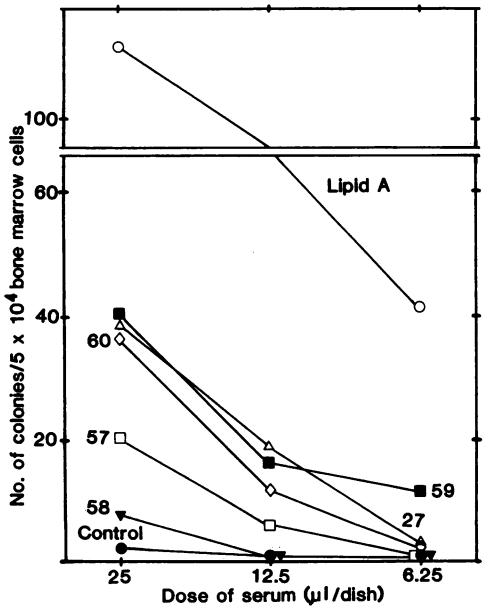

FIG. 2. CSF-inducing activity of chemically synthesized lipid A-subunit analogs with the (RS) stereospecificity. Natural lipid A (○), GLA-57 (□), GLA-27 (△), GLA-58 (▼), GLA-59 (■), or GLA-60 (◇) was administered i.v. at a dose of 10 μg per mouse. ●, Mock-treated control. The CSF activity induced in the serum was expressed as the number of colonies in the indicated dilution.

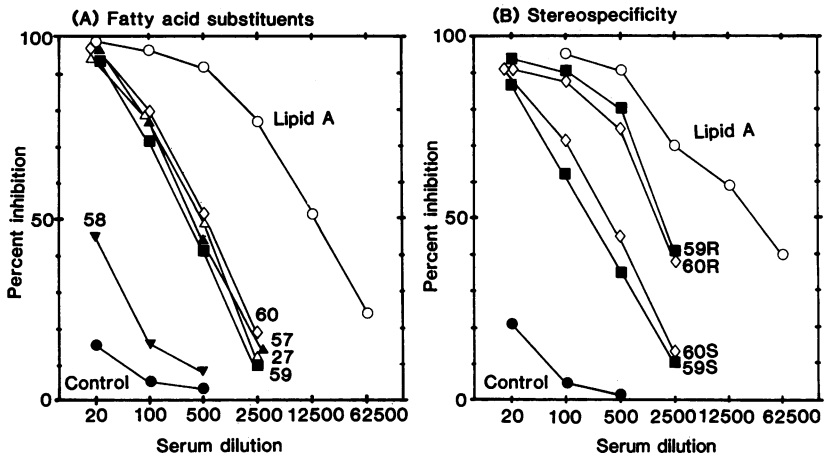

FIG. 3. TNF-inducing activity of chemically synthesized lipid A-subunit analogs with stereospecificity. Natural lipid A (○), GLA-57 (▲), GLA-27 (△), GLA-58 (▼), GLA-59 (■), or GLA-60 (◇) was administered i.v. at a dose of 10 μg per mouse. ●, Mock-treated control. TNF activity induced in the serum was expressed as the percent inhibition of [^{3}H]dThd uptake into L929 cells.

Adjuvant activity. Concomitant injection with the antigen and GLA-27 induced significant enhancement of the anti-BSA IgG antibody response, whereas the adjuvant activity of GLA-58 was very weak (Table 4). GLA-57 and GLA-59 were somewhat more active than GLA-27. Among compounds with the (RS) configuration, GLA-60 showed the strongest adjuvant activity. The adjuvant activity of GLA-59 and GLA-60 with the (RR) configuration was much stronger than that of compounds with the (SS) configuration.

DISCUSSION

In this paper we demonstrate clearly the importance of fatty acid substituents of lipid A-subunit analogs for the expression of immunopharmacological activities such as mediator (CSF and TNF) production and B-cell and macrophage activation. GLA-27 carrying 3-*O*-linked C_{14} and *N*-linked C_{14}-O-(C_{14}) groups showed significant activities without pyrogenicity and local Shwartzman reaction, though the activities were weaker than those of natural lipid A. GLA-57 carrying 3-*O*-C_{14} and *N*-C_{14}-O-(C_{12}) groups also exhibited activities equivalent to or somewhat weaker than those of GLA-27, while the activities of GLA-58 carrying 3-*O*-linked C_{14} and *N*-linked C_{14}-O-(C_{16}) groups were very low or undetectable. With other research groups we have shown that the immunopharmacological activities of synthetic *Salmonella*-type lipid A (compound 516 and LA-16-PP) are significantly less active than those of synthetic *E. coli* lipid A (506 and LA-15-PP) (2, 3, 7). The difference in the chemical structure between heptacyl 516 and hexaacyl 506 is the presence and absence of a C_{16} group bound to the hydroxy group of the 2-*N*-linked C_{14}OH group in the GlcN-I of *E. coli* lipid A. Galanos and colleagues suggested that the presence of seven fatty acids resulted in a different three-dimensional organization of the molecule and that the higher lipophilic character of 516, as compared with those of *Salmonella* lipid

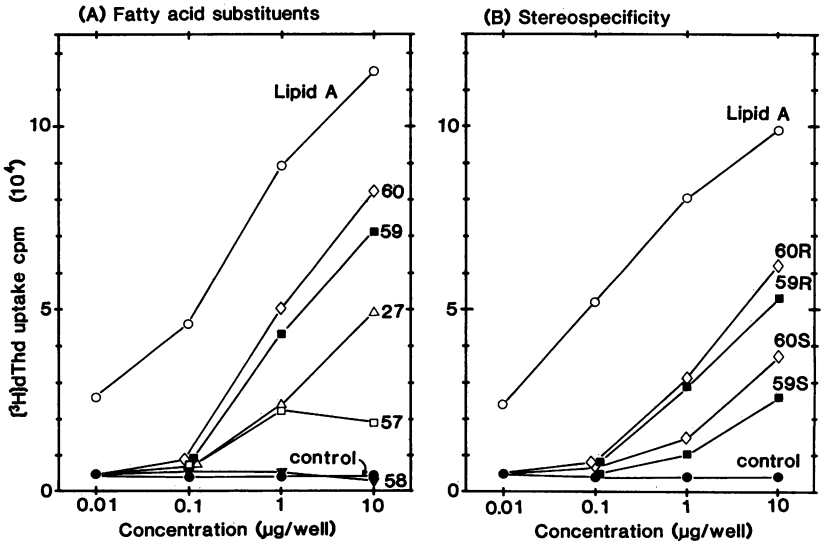

FIG. 4. Mitogenic activity of chemically synthesized lipid A-subunit analogs with stereospecificity. C3H/He spleen cells were stimulated for 48 h with the indicated dose of natural lipid A (○), GLA-57 (□), GLA-27 (△), GLA-58 (▼), GLA-59 (■), or GLA-60 (◇). ●, Mock control without test samples. The mitogenic activity is expressed as arithmetic mean [^{3}H]dThd uptake (counts per minute [cpm]) of triplicate cultures.

TABLE 2. PBA activity of chemically synthesized lipid A-subunit analog with streospecificity

Sample tested[a]	Anti-TNP-HRBC IgM PFC/culture mean ± SD (SI)[b]
Expt 1	
Mock control	86 ± 23
GLA-57 (RS)	442 ± 167 (5.1)
GLA-27 (RS)	438 ± 119 (5.1)
GLA-58 (RS)	60 ± 23 (0.7)
GLA-59 (RS)	518 ± 41 (6.0)
GLA-60 (RS)	503 ± 93 (5.8)
Lipid A	1,859 ± 295 (21.6)
Expt 2	
Mock control	106 ± 34
GLA-59 (RR)	1,073 ± 393 (10.1)
GLA-59 (SS)	555 ± 136 (5.2)
GLA-60 (RR)	1,167 ± 82 (11.0)
GLA-60 (SS)	398 ± 49 (3.8)
Lipid A	2,173 ± 253 (20.5)

[a] C3H/He spleen cells were incubated for 48 h with or without test samples. Dose of each sample was 10 μg per well.

[b] Plaque-forming cell (PFC) counts were determined using trinitrophenylated horse erythrocytes (TNP-HRBC) as indicator cells. Results are expressed as arithmetic mean ± standard deviation of triplicate cultures. Stimulation index (SI) was the value relative to the control.

A and compound 506, and its resulting lower solubility might be another reason for its reduced endotoxic potency (2). Although we need further study to clarify the physicochemical nature of these compounds, no difference in solubility was observed macroscopically among all synthetic compounds. Therefore, it may be concluded that the presence of the C_{16} group in an amide-bound acyloxyacyl group in some way reduces the potency of both monosaccharide and disaccharide analogs of lipid A. It would be very interesting to investigate whether or not GLA-58 can inhibit competitively the binding of lipid A to the receptor and prevent endotoxin shock.

It was found that GLA-59 and GLA-60, especially GLA-60 with the (RS) configuration, induced stronger B-cell activation and IgG antibody response against BSA than did GLA-27 with the (RS) configuration, though their mediator (CSF and TNF)-inducing activity and macrophage activation capacity were almost equivalent to those of GLA-27. GLA-60 (RS) isomer also exhibited stronger resistance against *Pseudomonas aeruginosa* and vaccinia virus infections than GLA-27 (RS) isomer (unpublished data). Moreover, GLA-60 was demonstrated to have stronger antitumor activity than GLA-27 against Meth A fibrosarcoma (M. Nakatsuka, Y. Kumazawa, J. Y. Homma, M. Kiso, and A. Hasegawa, submitted for publication). It therefore seems that the structure of GLA-60 is more relevant to the lipophilic character of the monosaccharide-type analog for expressing immunopharmacological activities, since GLA-60 carries a C_{14}-O-(C_{14}) group as the 3′-*O*-linked fatty acid substituent of the nonreducing sugar part (GlcN-II) and a C_{14}OH group as the 2-*N*-linked fatty acid substituent of the GlcN-I of *E. coli* lipid A (Fig. 1).

Among stereoisomers of GLA-59 and GLA-60, compounds with the (RR) configuration showed much higher TNF-inducing, B-cell and macrophage activating, and adjuvant activities than compounds with the (SS) configuration. In a previous study (6), we showed that the (S) isomers of GLA-27 and GLA-40 (1-deoxy derivative of GLA-27) exhibited higher TNF-inducing activity than did the (R) isomers. Although the results were reversed for TNF-inducing activity, the results for the (RR) isomers of GLA-59 and GLA-60 can be considered reasonable since fatty acid substituents in natural lipid A have the (RR) configuration.

With respect to CSF induction with test samples administered i.v. 6 h previously, GLA-27, GLA-59, and GLA-60 showed significant CSF-inducing activity, though the activity was weaker than that of natural lipid A (Fig. 2). CSF

TABLE 3. Potency of peritoneal macrophages stimulated in vivo with chemically synthesized lipid A-subunit analogs with stereospecificity[a]

Sample tested	Cell yield (10^6/mouse)	Phagocytic activity[b] (%)	Acid phosphatase activity[c] (mU)	Cytostasis-inducing activity[d] (%)
Expt 1				
Control	4.3 ± 0.6	1.9 ± 1.9	0.48 ± 0.07	7.4 ± 3.0
GLA-57 (RS)	9.6 ± 0.8 (2.2)*	3.0 ± 0.1 (1.6)**	0.77 ± 0.05 (1.6)**	31.6 ± 1.8**
GLA-27 (RS)	9.7 ± 0.4 (2.3)*	4.4 ± 0.2 (2.3)**	0.85 ± 0.04 (1.7)*	30.2 ± 0.9**
GLA-58 (RS)	5.4 ± 0.6 (1.3)†	1.8 ± 0.1 (0.9)†	0.50 ± 0.01 (1.0)†	18.3 ± 1.8*
GLA-59 (RS)	9.2 ± 1.3 (2.1)*	3.4 ± 0.2 (1.8)**	0.80 ± 0.08 (1.7)*	32.9 ± 4.2**
GLA-60 (RS)	9.9 ± 0.4 (2.3)**	4.0 ± 0.8 (2.1)*	0.76 ± 0.01 (1.7)*	34.1 ± 1.1**
Lipid A	13.8 ± 1.2 (3.2)**	5.2 ± 0.5 (2.7)**	1.12 ± 0.01 (2.3)**	98.6 ± 1.1***
Expt 2				
Control	3.8 ± 0.1	1.4 ± 0.1	0.39 ± 0.08	10.2 ± 2.4
GLA-59 (RR)	10.8 ± 1.3 (2.8)**	3.4 ± 0.4 (2.4)*	0.82 ± 0.03 (2.1)*	43.0 ± 21.8†
GLA-59 (SS)	6.1 ± 2.3 (1.6)†	2.1 ± 1.2 (1.5)†	0.55 ± 0.09 (1.4)†	10.5 ± 11.2†
GLA-60 (RR)	8.4 ± 0.6 (2.2)**	3.6 ± 0.4 (2.6)**	0.86 ± 0.13 (2.2)**	42.7 ± 17.3†
GLA-60 (SS)	5.0 ± 1.3 (1.3)†	1.9 ± 0.1 (1.4)*	0.56 ± 0.05 (1.4)†	16.1 ± 5.2†
Lipid A	13.9 ± 1.6 (3.7)	5.8 ± 1.0 (4.1)**	1.09 ± 0.02 (2.8)**	94.8 ± 0.8*

[a] Twenty micrograms of each sample was injected i.p. in a volume of 0.1 ml into three CDF_1 mice per group in each experiment. Four days later, peritoneal cells were recovered from the mice and pooled. Results are expressed as arithmetic mean ± standard deviation of two experiments carried out separately. Parentheses indicate stimulation index (see Table 2, footnote *b*). Statistic analyses were carried out by the Student *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; †, not significant).

[b] Phagocytic activity is expressed as mean percent phagocytosis ± standard deviation of two experiments carried out separately using ^{51}Cr-labeled, antibody-sensitized sheep erythrocytes.

[c] The cellular lysosomal enzyme (acid phosphatase) activity of lysates of 10^5 cells per well was determined at A_{405} using *p*-nitrophenyl phosphate as a substrate.

[d] Peritoneal macrophages (1.25×10^5 cells per well in 96-well microtiter plates) were cocultivated for 40 h with target EL-4 lymphoma cells in an effector/target cell ratio of 10:1. Values indicate the percentage of inhibition of [^{3}H]dThd uptake into the target cells.

TABLE 4. Adjuvant activity of chemically synthesized lipid A-subunit analogs with stereospecificity

Immunization with[a]:	Anti-BSA IgG antibody response[b] mean ± SD (SI)[b]
Expt 1	
BSA alone	17 ± 12
BSA + GLA-57	170 ± 26 (10.0)***
BSA + GLA-27	128 ± 58 (7.5)***
BSA + GLA-58	34 ± 9 (2.0)*
BSA + GLA-59	187 ± 52 (11.0)***
BSA + GLA-60	265 ± 113 (15.6)***
BSA + lipid A	382 ± 52 (22.5)***
Expt 2	
BSA alone	2 ± 3
BSA + GLA-59(RR)	435 ± 42 (217)***
BSA + GLA-59(SS)	172 ± 57 (86)***
BSA + GLA-60(RR)	454 ± 82 (227)***
BSA + GLA-60(SS)	236 ± 41 (118)***
BSA + lipid A	504 ± 96 (252)***

[a] Female 8-week-old CDF_1 mice (six per sample) were immunized i.p. with 10 μg of BSA together with (or without) 10 μg of test sample. The serum was separated from the blood of mice 25 days after immunization.

[b] The antibody titer is expressed as arithmetic mean enzyme-linked immunosorbent assay units ± standard deviation. For other details, see the footnotes to Table 3.

production in blood may result in increased numbers of granulocytes and monocytes (macrophages) in the periphery and induce enhanced nonspecific host resistance against some microbial infections, in which granulocytes and macrophages play the central role for the resistance in collaboration with complements and other humoral factors. In fact, i.p. administration of 10 μg of GLA-60 on the day before *P. aeruginosa* infection induced remarkable protection comparable to the host resistance elicited by the administration of 1 μg of natural lipid A (M. Nakatsuka, Y. Kumazawa, J. Y. Homma, M. Kiso, and A. Hasegawa, manuscript in preparation).

Raetz and colleagues separated biosynthetic monosaccharide-type precursors of lipid A, i.e., lipids X and Y, from a mutant of *E. coli* K-12 strain MN7 (18, 22) and showed that lipid X had no detectable endotoxic activity but was active in the *Limulus* test, was mitogenic for murine spleen cells, polyclonally activated murine B cells, and stimulated murine macrophages (17, 19). Recently, Takahashi and co-workers reported a comparative study on the activity of GLA-27 with lipid X in reference to endotoxic and other immunopharmacological activities (21). Among the endotoxic activities, significant lethal toxicity was found in galactosamine-sensitized mice when they were given 10 μg of GLA-27 i.v. but not lipid X at the same dose, whereas significant pyrogenicity was detected with lipid X at a dose of 10 μg/kg but not with GLA-27 even at a dose of 31.6 μg/kg. With respect to immunopharmacological activities, e.g., adjuvant activity in terms of enhancement of antibody response against sheep erythrocytes, TNF-inducing activity, ability to activate the clotting-enzyme cascade of *Tachypleus tridentatus* amoebocyte lysate, enhancing effect on the activation of guinea pig peritoneal macrophages in reference to O_2^- generation and [^{14}C]glucosamine uptake, and interleukin 1-inducing activity, GLA-27 exhibited much stronger activities than lipid X. These results suggest that the immunopharmacological activities of GLA-60 are much stronger than those of lipid X, since GLA-60 demonstrated stronger activities than GLA-27.

Natural lipid A induces toxic activities such as pyrogenicity, local Shwartzman activity, and lethal toxicity in galactosamine-sensitized mice, but the compounds in our investigation showed neither detectable pyrogenicity nor local Shwartzman activity (Table 1). However, the dose that induced weaker lethal toxicity in galactosamine-sensitized mice still remained at 1 μg per mouse. Recently, Lehmann et al. reported that TNF may be a mediator of lethal toxicity of endotoxin in galactosamine-sensitized mice (13). Further experiments are therefore necessary to clarify whether or not lethal toxicity induced by synthetic compounds is responsible for mediators produced in galactosamine-sensitized mice.

In conclusion, we have demonstrated in this study the existence of monosaccharide-type compounds, especially GLA-59 and GLA-60 with the (RR) configuration, possessing stronger immunostimulating and lesser toxic activities. We are now studying and evaluating the ability of these lipid A-subunit analogs as a new type of biological response modifier.

ACKNOWLEDGMENTS

This work was supported in part by a grant-in-aid for scientific research from the Japanese Ministry of Education, Science and Culture (no. 60108003).

LITERATURE CITED

1. **Apte, R. N., C. Galanos, and D. H. Pluznik.** 1976. Lipid A, the active part of bacterial endotoxins in including serum colony stimulating activity and proliferation of splenic granulocyte/macrophage progenitor cells. J. Cell. Physiol. **87**:71–78.
2. **Galanos, C., O. Lüderitz, M. Freudenberg, L. Brade, U. Schade, E. T. Rietschel, S. Kusumoto, and T. Shiba.** 1986. Biological activity of synthetic heptacyl lipid A representing a component of *Salmonella minnesota* R595 lipid A. Eur. J. Biochem. **160**:55–59.
3. **Kanegasaki, S., K. Tanamoto, T. Yasuda, J. Y. Homma, M. Matsuura, M. Nakatsuka, Y. Kumazawa, A. Yamamoto, T. Shiba, S. Kusumoto, M. Imoto, H. Yoshimura, and T. Shimamoto.** 1986. Structure-activity relationship of lipid A: comparison of biological activities of natural and synthetic lipid A's with different fatty acid compositions. J. Biochem. (Tokyo) **99**:1203–1210.
4. **Kiso, M., H. Ishida, and A. Hasegawa.** 1984. Synthesis of biologically active, novel monosaccharide analogs of lipid A. Agric. Biol. Chem. **48**:251–252.
5. **Kiso, M., S. Tanaka, M. Fujita, Y. Fujishima, Y. Ogawa, and A. Hasegawa.** 1987. Synthesis of nonreducing-sugar subunit analogs of bacterial lipid A carrying an amide-bound (*3R*)-3-acyloxytetradecanoyl group. Carbohydr. Res. **162**:247–256.
6. **Kiso, M., S. Tanaka, M. Fujita, Y. Fujishima, Y. Ogawa, H. Ishida, and A. Hasegawa.** 1987. Synthesis of the optically active 4-*O*-phosphono-D-glucosamine derivatives related to the nonreducing sugar subunit of bacterial lipid A. Carbohydr. Res. **162**:127–140.
7. **Kotani, S., H. Takada, I. Takahashi, M. Tsujimoto, T. Ogawa, T. Ikeda, K. Harada, H. Okamura, T. Tamura, S. Tanaka, T. Shiba, S. Kusumoto, M. Imoto, H. Yoshimura, and N. Kasai.** 1986. 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. Infect. Immun. **52**:872–884.
8. **Kumazawa, Y., S. Ikeda, H. Takimoto, C. Nishimura, M. Nakatsuka, J. Y. Homma, M. Kiso, and A. Hasegawa.** 1987. Effect of stereospecificity of chemically synthesized lipid A-subunit analogues GLA-27 and GLA-40 on the expression of immunopharmacological activities. Eur. J. Immunol. **17**:663–667.
9. **Kumazawa, Y., M. Matsuura, J. Y. Homma, Y. Nakatsuru, M. Kiso, and A. Hasegawa.** 1985. B cell activation and adjuvant activities of chemically synthesized analogues of the nonreduc-

ing sugar moiety of lipid A. Eur. J. Immunol. **15**:199–201.

10. **Kumazawa, Y., M. Matsuura, T. Maruyama, J. Y. Homma, M. Kiso, and A. Hasegawa.** 1986. Structural requirements for inducing in vitro B lymphocyte activation by chemically synthesized derivatives related to the nonreducing D-glucosamine subunit of lipid A. Eur. J. Immunol. **16**:1099–1103.
11. **Kumazawa, Y., M. Matsuura, Y. Nakatsuru-Watanabe, M. Fukumoto, C. Nishimura, J. Y. Homma, M. Inage, S. Kusumoto, and T. Shiba.** 1984. Mitogenic and polyclonal B cell activation activities of synthetic lipid A analogues. Eur. J. Immunol. **14**:109–114.
12. **Lasfargues, A., D. Charon, F. Tarigalo, A. Ledur, L. Szabo, and R. Chaby.** 1986. Analysis of the lipopolysaccharide-inducing cytostatic activity of macrophages, by the use of synthetic models. Cell. Immunol. **98**:8–17.
13. **Lehmann, V., M. A. Freudenberg, and C. Galanos.** 1987. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. J. Exp. Med. **165**:657–663.
14. **Matsuura, M., Y. Kojima, J. Y. Homma, Y. Kubota, A. Yamamoto, M. Kiso, and A. Hasegawa.** 1984. Biological activities of chemically synthesized analogues of the nonreducing sugar moiety of lipid A. FEBS Lett. **167**:226–230.
15. **Matsuura, M., Y. Kojima, J. Y. Homma, Y. Kumazawa, A. Yamamoto, M. Kiso, and A. Hasegawa.** 1986. Effect of backbone structure stereospecificities of lipid A-subunit analogues on their biological activities. J. Biochem. (Tokyo) **99**:1377–1384.
16. **Matsuura, M., A. Yamamoto, Y. Kojima, J. Y. Homma, M. Kiso, and A. Hasegawa.** 1985. Biological activities of chemically synthesized partial structure analogues of lipid A. J. Biochem. (Tokyo) **98**:1229–1237.
17. **Nishijima, M., F. Amano, Y. Akamatsu, K. Akagawa, T. Tokunaga, and C. R. H. Raetz.** 1985. Macrophage activation by monosaccharide precursors of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **82**:282–286.
18. **Nishijima, M., and C. R. H. Raetz.** 1981. Characterization of two membrane-associated glycolipids from an *Escherichia coli* mutant in phosphatidylglycerol. J. Biol. Chem. **256**:10690–10696.
19. **Raetz, C. R. H., S. Purcell, and K. Takayama.** 1983. Molecular requirements for B-lymphocyte activation by *Escherichia coli* lipopolysaccharide. Proc. Natl. Acad. Sci. USA **80**:4624–4628.
20. **Shimizu, T., S. Akiyama, T. Masuzawa, Y. Yanagihara, S. Nakamoto, T. Takahashi, K. Ikeda, and K. Achiwa.** 1985. Antitumor activity and biological effects of chemically synthesized monosaccharide analogues of lipid A in mice. Chem. Pharm. Bull. **33**:4621–4624.
21. **Takahashi, I., S. Kotani, H. Takada, M. Tsujimoto, T. Ogawa, T. Shiba, S. Kusumoto, M. Yamamoto, A. Hasegawa, M. Kiso, M. Nishijima, F. Amano, Y. Akamatsu, K. Harada, S. Tanaka, H. Okamura, and T. Tamura.** 1987. Requirement of a properly acylated β(1→6)-D-glucosamine disaccharide bisphosphate structure for efficient manifestation of full endotoxic and associated bioactivities of lipid A. Infect. Immun. **55**:57–68.
22. **Takayama, K., N. Qureshi, P. Mascagni, M. A. Nashed, L. Anderson, and C. R. H. Raetz.** 1983. Fatty acid derivatives of glucosamine-1-phosphate in *Escherichia coli* and their relation to lipid A. J. Biol. Chem. **258**:7379–7385.