INFECTION AND IMMUNITY, May 1994, p. 1705–1709
0019-9567/94/$04.00+0

Vol. 62, No. 5

Free Hydroxyl Groups Are Not Required for Endotoxic Activity of Lipid A

KEN-ICHI TANAMOTO*

Division of Microbiology, National Institute of Health Sciences, Kamiyoga 1-18-1 Setagaya-ku, Tokyo 158, Japan

Received 29 November 1993/Returned for modification 5 January 1994/Accepted 3 February 1994

Previous studies demonstrated that lipid A from *Salmonella abortusequi* loses its B-cell mitogenicity for murine spleen cells as a result of the introduction of succinyl residues on hydroxyl groups and that the inactivated lipid A specifically antagonizes the mitogenicity of endotoxin. Hypothesizing that the hydroxyl groups are essential both for its biological activity and for producing nontoxic preparations having antagonistic activity, I tested the role of the hydroxyl groups in its activities by using well-characterized biologically active lipid A preparations synthesized chemically (*Escherichia coli* and *Salmonella* types 506 and 516, respectively) by the introduction of either succinyl or acetyl residues at the hydroxyl groups of each of these lipid A preparations. However, the biological activities of neither lipid A preparation were reduced at all after succinylation; in fact, succinylated 516 became much more potent than the original molecule with respect to most activities tested, i.e., lethal toxicity, *Limulus* gelation activity, and the induction of tumor necrosis factor release. On the other hand, when the hydroxyl groups were replaced with acetyl residues, the lethality and tumor necrosis factor-inducing activity of both lipid A preparations were decreased, whereas their *Limulus* gelation activity was increased. Mitogenicity was not affected much by the chemical modifications of either lipid A preparation. These findings indicate that although the residues introduced into the free hydroxyl groups of lipid A modulate its activities, the hydroxyl groups in lipid A need not exist in free form.

Endotoxin is implicated in severe clinical disorders caused by gram-negative bacterial infections (1, 12, 15). Chemically, endotoxin is a lipopolysaccharide (LPS), and the lipid moiety, lipid A, is known to be the active center of the endotoxin, which exerts numerous biological activities both in vivo and in vitro (11). The chemical structure of *Escherichia coli* lipid A was determined with the help of chemically synthesized derivatives and consists of a diglucosamine backbone with substitutions of two phosphate and six fatty acid molecules (4, 6). The relationship between the chemical structure of lipid A and its endotoxic activity has been intensively studied by several investigators using both natural LPS and synthetic lipid A analogs. In general, these workers have come to a tentative conclusion, i.e., that the disaccharide preparation is more active than the monosaccharide one, especially in toxic action. The phosphates in both positions 1 and 4′ are necessary for the manifestation of complete activity. The number and the position of the substituted fatty acids are also important to its activities. Overacylation of hydroxyl groups in lipid A decreases its activities, as shown for *Salmonella* lipid A (3, 7, 8, 16, 19, 20).

In another study, a colleague and I found that lipid A from *Salmonella abortusequi* loses its mitogenicity for murine spleen cells as a result of the introduction of succinyl residues at the hydroxyl groups of lipid A and that the inactivated lipid A specifically antagonizes the mitogenicity of endotoxin in a dose-dependent manner (17). These observations suggest that some free hydroxyl groups of lipid A are essential for its endotoxic activity and that substitution of the hydroxyl groups of lipid A is important for changing the endotoxically active lipid A structure not only to an inactive form but also to an antagonist of LPS. Both to confirm this hypothesis and to produce nontoxic preparations having antagonistic activity, I expanded my observations by using chemically synthesized pure lipid A preparations to simplify interpretation. Since the *S. abortusequi* lipid A used in the previous study is a mixture of many different structures and is known to contain two typical well-defined lipid A preparations, i.e., those of *E. coli* and salmonellae (Fig. 1), I used these two biologically active materials synthesized chemically. Here I show that, surprisingly, these active lipid A preparations completely retained their biological activities after succinylation of the free hydroxyl groups, indicating that hydroxyl groups need not exist in free form for these molecules to exert their endotoxic activities.

MATERIALS AND METHODS

Materials. Synthetic lipid A analog 516 (*Salmonella* lipid A) was a gift from Daiichi Kagaku Co., Ltd. (Tokyo, Japan). Synthetic complete lipid A 506 (*E. coli* lipid A) was purchased from Daiichi Kagaku Co. Recombinant tumor necrosis factor alpha (TNF-α) standards and rabbit polyclonal antisera against murine TNF-α were obtained from Asahi Kasei Kogyo, Ltd. RPMI 1640 medium with glutamine and Iscove's modified Dulbecco medium were products of GIBCO Laboratories, Grand Island, N.Y. Galactosamine was purchased from Sigma Chemical Co., St. Louis, Mo. Quantitative *Limulus* assay reagent (Endospecy) was obtained from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Pyrogen-free water was a product of Hikari Seiyaku Co. Ltd. (Tokyo, Japan).

Chemical modification. Succinylation was performed as described previously (14). In brief, a suspension containing 5 mg of each lipid A preparation (dried over P_2O_5 in a desiccator), 200 mg of succinic acid anhydride, and 300 μl of pyridine (dried with molecular sieves; Wako Chemical Co., Ltd.) was heated in a sealed tube at 60°C for 3 h. The mixture was poured into water (2 ml, 4°C), dialyzed, and lyophilized. Acetyl residues were introduced as follows. Five milligrams of each

* Mailing address: Division of Microbiology, National Institute of Health Sciences, Kamiyoga 1-18-1 Setagaya-ku, Tokyo 158, Japan. Phone: 03-3700-1141. Fax: 03-3707-6950.

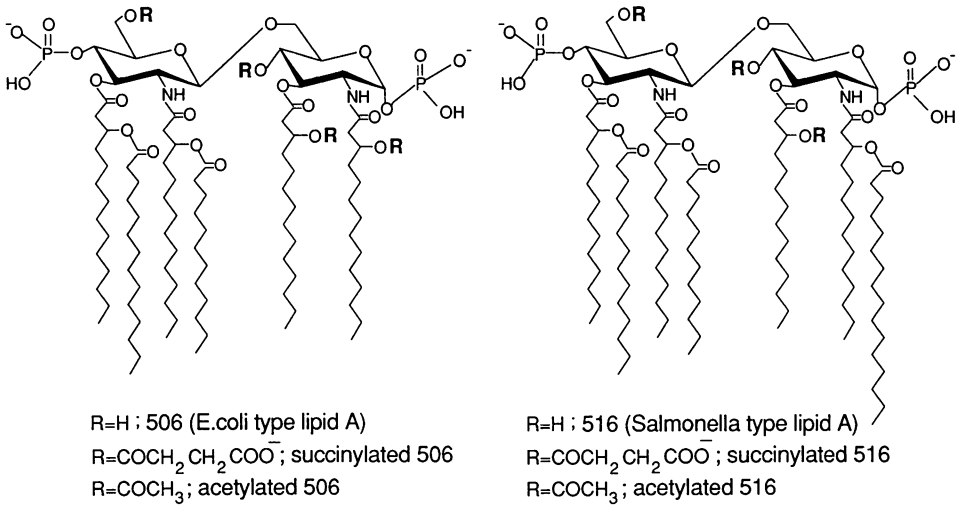

FIG. 1. Structures of *E. coli* and *Salmonella* lipid A molecules synthesized chemically (506 and 516, respectively) and their succinylated and acetylated derivatives. Succinyl and acetyl residues were introduced at the free OH groups of each of the lipid A preparations as described in Materials and Methods.

lipid A preparation was dissolved in a solution containing acetic acid anhydride and pyridine (0.2 ml each) in the presence of a small amount of dimethylaminopyridine. The mixture was allowed to stand overnight and then was dialyzed and lyophilized.

Mass spectrometry. Liquid secondary-ion mass spectrometry (LSI/MS) was performed on a VG ZAB-2SEQ spectrometer (VG Analytical) operated at 8 kV in the negative mode. The cesium gun was operated at 30 kV. Current-controlled scans were acquired at a rate of 30 s per decade. A mixture of ethanolamine and *m*-nitrobenzylalcohol (1:1) was used as the matrix.

Chemical analysis of succinylated preparations. Succinic acid introduced into lipid A preparations was determined as described below with glutaric acid as the internal standard. Each succinylated preparation, 0.3 mg, was dissolved in 4 M HCl and hydrolyzed for 3 h at 100°C. The reaction mixture was then evaporated by blowing nitrogen gas and dissolved in 1 ml of distilled water. A 100-μl sample of the solution was injected into a high-performance liquid chromatograph (Hitachi model L-6200) equipped with an octyldecyl silane reverse-phase column (C18) (5 μm; 4 by 250 mm) containing Lichrosorb. Elution was performed with distilled water containing 0.1% phosphoric acid at a flow rate of 0.5 ml/min. Free succinic acid was detected by measuring the A_{210} (UV monitor; Hitachi model L-4200).

Mitogenicity assay. For the mitogenicity assay (16), spleen cells from female BALB/c and C3H/HeJ mice (6 to 10 weeks old) were pressed through a wire grid and suspended in serum-free Iscove's modified Dulbecco medium containing L-glutamine and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (GIBCO) and supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were washed with the medium three times and then were cultured in Iscove's medium on microplates (Corning 25850-96) at 37°C in a humidified environment in the presence of 5% CO_2. Each well contained 8×10^5 cells in 200 μl of medium and a mitogen. After 48 h of incubation, [^{3}H]thymidine (0.2 μCi [7.4 kBq] per culture; Amersham) was added. After a further 24 h of incubation, cultures were harvested, collected on glass fiber filters, and washed with distilled water. The filters were dried and transferred to scintillation vials. Xylol-based scintillation fluid (5 ml) was added, and radioactivity was measured in a scintillation counter. The results are expressed as mean counts per minute of triplicate determinations. Standard errors were less than 10% in the mitogenicity assay and are not indicated in the figures.

Induction of TNF-α release from mouse peritoneal macrophages. Mouse peritoneal macrophages were obtained by washing the peritoneal cavity of female BALB/c mice (6 to 10 weeks old) with 5 ml of Iscove's medium (18). The cell number was adjusted to 2×10^6 cells per ml. One-milliliter aliquots of a cell suspension in Iscove's medium were cultured in 24-well Costar (Cambridge, Mass.) plates at 37°C with 5% CO_2 for 3 h, and the macrophages were allowed to adhere to the plates. After the cells had been washed three times with phosphate-buffered saline (PBS), 1 ml of Iscove's medium was added to each well and the cells were incubated with a test sample for 6 h. The supernatant of each culture was transferred to a plastic tube, the cells were centrifuged, and the supernatant was stored at −80°C until used for the determination of TNF-α production.

TNF-α assay. The TNF-α produced was determined by an L929 cytotoxicity assay. L929 murine fibroblasts were grown in tissue culture flasks containing RPMI 1640 medium supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 5 mM HEPES, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were detached with trypsin, washed, and resuspended in medium at 4×10^5 cells per ml, and 100-μl aliquots were plated in 96-well flat-bottom plates (Corning 25860-96). After incubation for 3 to 5 h at 37°C in 5% CO_2, 50 μl of actinomycin D (4 μg/ml) in RPMI 1640 medium was added to each well, as was 50 μl of a test sample (final volume, 200 μl per well). The results are expressed as means of triplicate determinations.

Lethal toxicity test. The lethality test was performed by the method described by Galanos et al. (2) with female C57BL/6 mice more than 10 weeks old and obtained from Nihon SLC (Hamamatsu, Japan). Test samples in 0.1 ml of pyrogen-free water were injected intravenously immediately after the intraperitoneal administration of 12 mg of D-galactosamine in 0.5 ml of pyrogen-free PBS.

***Limulus* amoebocyte gelation assay.** The activation of the proclotting enzyme of the horseshoe crab, *Limulus polyphemus*, was tested by a quantitative assay (Endospecy). Pyrogen-free water was used to dilute the test samples. The assay was performed with 96-well Costar flat-bottom plates at 37°C for 30 min, and the chromogen was measured at 550 nm with a microplate reader (Molecular Devices).

RESULTS

Succinylation and acetylation of synthetic lipid A preparations. Mass spectrometry of 506 after succinylation and acetylation showed that three or four molecules of succinyl and acetyl residues (and, less frequently, two molecules) had been substituted for the four free hydroxyl groups of 506. For 516, two or three molecules of both residues (and, less frequently, one molecule) were introduced into the three free hydroxyl groups. No apparent degradation products or dephosphorylated or deacylated forms were observed in the spectra of either sample, indicating that no degradation, such as dephosphorylation or deacylation, occurred during the course of chemical modification. The number of succinyl residues introduced into 506 and 516 was estimated by measuring free succinic acid after hydrolysis of the sample with 4 N HCl, with glutaric acid as the internal standard. Succinylation resulted in the introduction of 3.56 and 2.75 mol of succinyl residues into the lipid A preparations, respectively, providing quantitative confirmation of the results of mass spectrometry.

Mitogenicity of 506, 516, and their succinylated and acetylated derivatives. The mitogenic activity of both lipid A preparations and their succinylated and acetylated derivatives was tested on murine splenic cells. As shown in Fig. 2A, the mitogenicity of 506 remained at almost comparable levels after both succinylation and acetylation. Succinylated 506, however, exhibited significantly higher activity than either 506 or acetylated 506 at higher concentrations (>10 μg/ml). No significant difference was observed between 516 and its derivatives (Fig. 2B). The number of background counts per minute with no lipid A was 530 ± 35 (mean ± standard deviation in triplicate experiments). These two lipid A preparations and their derivatives did not stimulate spleen cells from non-endotoxin-responsive C3H/HeJ mice (data not shown).

Induction of TNF-α release by 506, 516, and their succinylated and acetylated derivatives. TNF-α released from stimulated peritoneal macrophages of BALB/c mice into the medium was estimated by measuring cytotoxicity against actinomycin D-sensitized L929 murine fibroblasts. As shown in Fig. 3A, the cells started to secrete TNF-α at a concentration of 1 ng of 506 per ml. The induction of TNF-α release from macrophages by 506 increased dose dependently, and maximum TNF-α production (34 ng/ml) was observed at the highest concentration tested, 10 μg/ml. The activity of 506 remained at comparable levels when succinyl residues were introduced into the hydroxyl groups but decreased significantly after acetylation (Fig. 3A). The activity of 516 was about 10 times lower than that of 506 at the minimum stimulatory doses. Its activity, however, was significantly increased by succinylation but was drastically reduced by acetylation, as shown in Fig. 3B.

No induction of TNF-α release by either of the lipid A preparations or their derivatives was observed when peritoneal macrophages from C3H/HeJ mice were used.

To confirm that the cytotoxic activity against L929 cells was due to TNF-α, aliquots of supernatants from macrophage cultures were incubated for 12 h with polyclonal rabbit antiserum to TNF-α, with nonspecific immunoglobulin G as the control. The polyclonal antiserum to TNF-α completely abolished the cytotoxicity of the supernatants stimulated with all the samples tested.

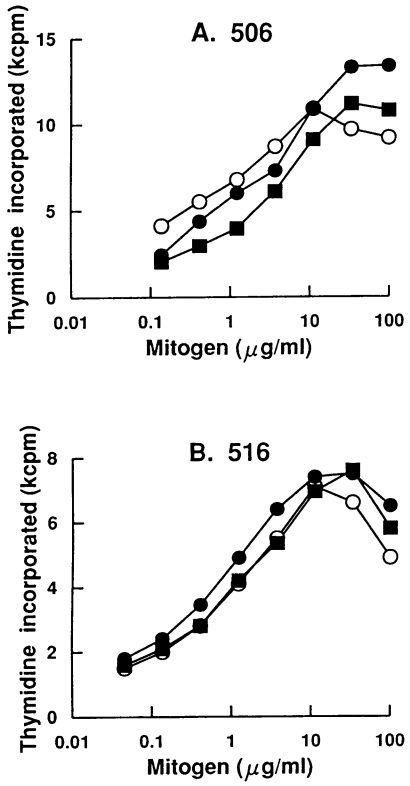

FIG. 2. Mitogenic responses of murine spleen cells to 506 and 516 and their derivatives. Murine spleen cells, 8×10^5 cells per well, were cultured with various concentrations of test samples for 24 h. Radioactivity ([^{3}H]thymidine) incorporated during an additional 24 h of incubation was measured. The results are expressed as mean counts per minute in triplicate experiments. The number of background counts per minute with no lipid A was 530 ± 35 (mean ± standard deviation). Symbols: ○, original; ●, succinylated; ■, acetylated.

Lethal toxicity of 506, 516, and their succinylated and acetylated derivatives. The lethal toxicity of the samples was tested with galactosamine-sensitized mice. The results are shown in Table 1. In this system, 506 exhibited 90% lethality at 10 ng per mouse, and moderate lethality was observed at 1 ng per mouse. Succinylated 506 was also lethal at 1 ng per mouse, and at 10 ng per mouse it was 100% lethal. On the other hand, the lethality of acetylated 506 at 10 ng per mouse was 20%, and no lethality was observed at 1 ng per mouse, indicating that the toxicity of 506 was reduced at least 10-fold by acetylation. For 516, 100% lethality was first observed at 100 ng per mouse. Its activity was increased significantly by succinylation: 50% lethality was exhibited at 10 ng per mouse. On the other hand, the lethality of 516 was decreased by acetylation: 67% lethality was expressed at 100 ng per mouse.

Chromogenic *Limulus* amoebocyte lysate testing of 506, 516, and their succinylated and acetylated derivatives. Synthetic *E. coli* lipid A 506 activated the cascade of the clotting system of the horseshoe crab at a concentration of several picograms per milliliter, and linear dose dependency was obtained up to 100 pg/ml. Both succinylated and acetylated 506 expressed higher activity than the original 506, and linear dose dependency was obtained up to 12.5 and 25 ng/ml, respectively (Fig. 4A). On the other hand, synthetic *Salmonella* lipid A 516 showed less

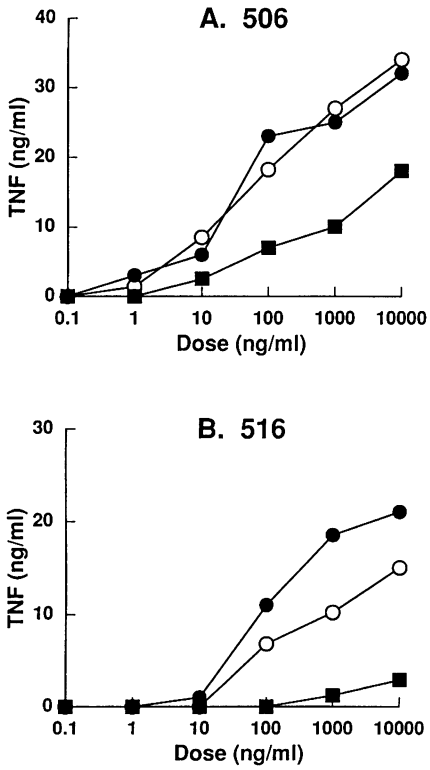

FIG. 3. Induction of TNF-α (TNF) release from peritoneal macrophages of BALB/c mice by 506, 516, and their derivatives. Murine peritoneal macrophages, 2×10^6 cells per ml, were cultured with various test sample concentrations for 6 h, and the supernatants were assayed for TNF-α. The results are expressed as means in triplicate experiments. Symbols are as defined in the legend to Fig. 2.

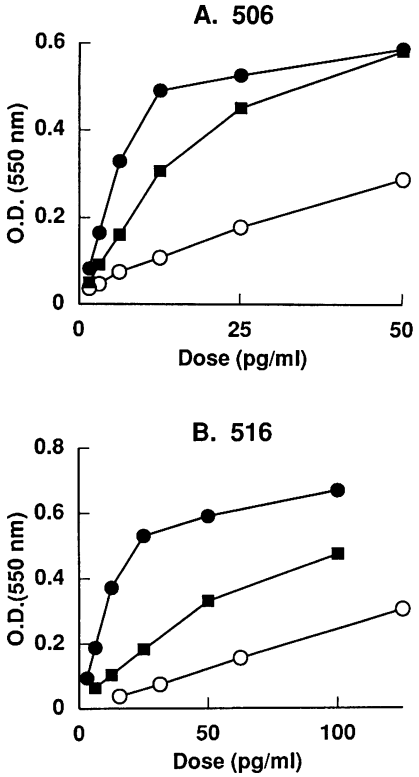

FIG. 4. *Limulus* gelation activity of 506, 516, and their derivatives. *Limulus* amoebocyte lysate was incubated with test samples for 30 min, and the chromogen released was measured. Symbols are as defined in the legend to Fig. 2. O.D., optical density.

activity than 506. Its activity, however, was increased by both succinylation and acetylation. The same levels of chromogenicity (optical density, 0.2) were obtained at 100 pg of 516 per ml, 25 pg of acetylated 516 per ml, and 6.25 pg of succinylated 516 per ml (Fig. 4B).

DISCUSSION

Recently, the antagonism of endotoxic activity by nontoxic LPS (or lipid A) and its analogs has been studied intensively, and some of the nontoxic lipid A preparations or their analogs have been found to act as LPS antagonists (5, 9, 10, 13, 16).

TABLE 1. Lethal toxicity of 506, 516, and their derivatives for galactosamine-sensitized mice[a]

Lipid A prepn	No. of dead mice/total no. tested at the following dose (μg/mouse):				
	0.001	0.03	0.01	0.1	1
506	5/15	4/6	9/10		
Succinylated 506	3/15	6/6	8/8		
Acetylated 506	0/6		2/10	6/6	
516			0/6	6/6	
Succinylated 516	0/6		4/8	6/6	
Acetylated 516			0/6	4/6	4/4

[a] Test samples in 0.1 ml of pyrogen-free water were injected intravenously, immediately after intraperitoneal administration of 12 mg of D-galactosamine in 0.5 ml of pyrogen-free PBS.

Since antagonistic activity was generally seen with lipid A or its analogs which do not express endotoxicity, one of the most promising strategies for searching for such antagonists systematically is chemical modification of active lipid A. In a previous study, a colleague and I found that succinylated lipid A from *S. abortusequi* is a potent and specific antagonist of endotoxic mitogenicity and expresses inhibitory activity dose dependently and competitively (17). Such antagonism was also observed with succinylated lipid A from other sources (unpublished data). These findings suggest that the hydroxyl groups in the active lipid A structure play an important role in the manifestation of endotoxic activity and that modification of the hydroxyl groups is involved in antagonistic activity. In the present study, I performed chemical modifications with the aim of clarifying the role of the hydroxyl groups in the activities of lipid A, as well as investigating correlations between the chemical structure and biological activities of lipid A. Since the *S. abortusequi* lipid A used in the previous study is a mixture of many different structures and is known to contain two typical, well-characterized lipid A preparations (*E. coli* and *Salmonella* types 506 and 516), I used these two chemically synthesized biologically active lipid A preparations. Chemical modification by both succinylation and acetylation was performed effectively, as seen from the results of LSI/MS, although some free hydroxyl groups still remained in both lipid A preparations. The results of testing the biological activities of modified lipid A showed, surprisingly, that neither lipid A preparation had lost its biological activities as a result of succinylation and even that succinylated 516 had enhanced potency when tested for lethal toxicity, tumor necrosis factor-inducing activity, and

Limulus gelation activity. These findings indicate that the hydroxyl groups in lipid A need not exist in free form. On the other hand, when the hydroxyl groups were substituted for by nonanionic and hydrophobic acetyl residues, the lethality and tumor necrosis factor-inducing activity of both lipid A preparations decreased, while their *Limulus* gelation activity increased. Mitogenicity was not significantly affected by these chemical modifications of either lipid A preparation. The relatively low levels of activity of acetylated lipid A may be partly explained by the lower solubility of the preparation caused by the introduction of the more hydrophobic residues. These findings indicate that chemical modifications modulate lipid A activity according to the residues introduced at the hydroxyl groups but that lipid A does not lose its activities completely as a result of these modifications and, hence, does not become an antagonist of lipid A effects. The antagonistic structure which had been suggested to exist in the mixture of succinylated *S. abortusequi* lipid A in a previous study was revealed to be different from those tested in the present study, and the unknown antagonist in the mixture may suppress the activities of the active preparations. The antagonistic structure is now being intensively studied; one candidate, lipid A precursor, has been found to lose its biological activities completely after succinylation, and the succinylated precursor antagonizes LPS effects (unpublished data).

The results of this study show that the biological activities of chemically modified lipid A are modulated by the nature of the substituents introduced at the hydroxyl groups and that the free hydroxyl groups of lipid A can be replaced by other residues without a loss of biological activities. They provide important information for the meaning of the relationship between the chemical structure and biological activities of lipid A.

ACKNOWLEDGMENT

The skillful technical assistance of Y. Haishima in performing LSI/MS is greatly appreciated.

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