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Predominant role of the substituents on the hydroxyl groups of 3-hydroxy fatty acids of non-reducing glucosamine in lipid A for the endotoxic and antagonistic activity

Ken-ichi Tanamoto*

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

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Abstract The synthetic disaccharide precursor of lipid A (406: identical to lipid IV_A) was found to reduce its endotoxic activity in mice by an order of 10^5 or more, by replacing the hydroxyl groups with succinyl or acetyl residues. Both the succinylated and acetylated 406 were also found to antagonize the endotoxic mitogenicity on murine splenocytes. Previous studies demonstrated that the succinylated or acetylated synthetic complete lipid A preparations retained the whole endotoxic activity [1994, Infect. Immunol. 62, 1705]. The drastic contrast in all of these results suggests the importance of the substituents on the hydroxyl groups of 3-hydroxy fatty acids of non-reducing glucosamine of lipid A for the activity and for transformation to the antagonistic structure.

Key words: Endotoxin; Synthetic lipid A; Lipid A precursor; Non-toxic lipid A; Endotoxin antagonist

1. Introduction

Lipid A is the active center of endotoxin which exerts numerous biological activities both in vivo and in vitro [1,2]. The usual lipid A consists of a diglucosamine backbone with substitution of phosphate and fatty acid molecules. The relationship between the chemical structure of lipid A and its endotoxic activity has been studied by many investigators. A general concept of the relationship has been obtained, i.e. the preparations with the disaccharide backbone exhibit higher activity than those with the monosaccharide backbone, especially in toxicity. Both of the two phosphates in positions 1 and 4′ play an important role in the manifestation of the full activity [3,4]. Fatty acids especially play an essential role in the activity. Their number, binding site and the kind appear to be critical determinants of the capacity for the activity [5–7]. In fact, some of the non-toxic lipid A preparations so far found have the characteristic fatty acids with the usual diglucosamine backbone and phosphates [8–10]. Since the hydroxyl groups in lipid A act as the acceptors of the fatty acids, it seemed interesting to examine the role of free hydroxyl groups, or the substitution of the residues.

The chemically synthesized disaccharide lipid A precursor 406 (identical to lipid IV_A or precursor Ia) has been shown, and was confirmed in this study, to be biologically active in mice [11,12]. It contains 4 free OH groups of 3-hydroxy myristic acid, some of which are substituted by fatty acids to form the complete lipid A (Fig. 1). In the previous study, I showed that the free hydroxyl groups in the complete lipid A preparations (*E. coli* and *Salmonella* types) are not required for the endotoxic activity [13]. I now show that, in contrast, the lipid A precursor 406 lost all of its biological activity completely after substitution of free hydroxyl residues, and most importantly, these chemically modified 406 preparations proved to be potent antagonists of endotoxin in mice. I, hence, propose that the substituents play a predominant role in both changing the endotoxically active lipid A structure to an inactive form and into an antagonist of endotoxin.

2. Materials and methods

2.1. Materials

Synthetic lipid A analogues 406 was a gift of Daiichi Kagaku Co. Ltd. (Tokyo). Synthetic complete lipid A (*E. coli* type; 506) was purchased from Daiichi Kagaku Co. Ltd. Lipopolysaccharide (LPS) was extracted from *S. abortus equi* by the aqueous phenol method [14]. Lipid A was obtained as an insoluble substance after treatment of LPS with 1% acetic acid at 100°C for 90 min [15]. Iscove's modified Dulbecco medium containing L-glutamine and HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid) buffer and RPMI 1640 medium were from GIBCO Laboratories (Grand Island, NY). Recombinant TNFα standards and rabbit polyclonal antisera against murine TNFα were obtained from Asahi Kasei Kogyo, Ltd. (Japan).

2.2. Chemical modifications

2.2.1. Succinylation of 406 and its chemical analysis [16,17]. A suspension of 5 mg of 406, 200 mg succinic anhydride, and 300 μl of pyridine was heated in a sealed tube at 60°C for 3 h. Succinic acid introduced into 406 was liberated by hydrolysis with 4 M HCl for 3 h at 100°C and was determined by high-performance liquid chromatograph (HPLC; Hitachi model L-6200) equipped with an ODS reverse-phase column (C18) (5 μm, 4 × 250 mm) containing Lichrosorb, using glutaric acid as the internal standard.

2.2.2. Acetylation of 406. Five mg of 406 was dissolved in a solution of 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, then dialyzed and lyophilized.

2.3. Mass spectrometry

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

2.4. Assay of endotoxic activity

2.4.1. Mitogenicity assay [18]. Spleen cells (8×10^5 cells/0.2 ml/well) of serum-free Iscove's medium) obtained from 6–10-week-old female BALB/C, and C3H/HeJ mice were incubated with mitogen at 37°C for 48 h. After a further 24 h incubation with [^{3}H]thymidine (0.2 μCi (7.4 kBq) per culture; Amersham), radioactivity incorporated into the cells was measured. The results are expressed as mean cpm of

*Corresponding author. Fax: (81) (3) 3707 6950.

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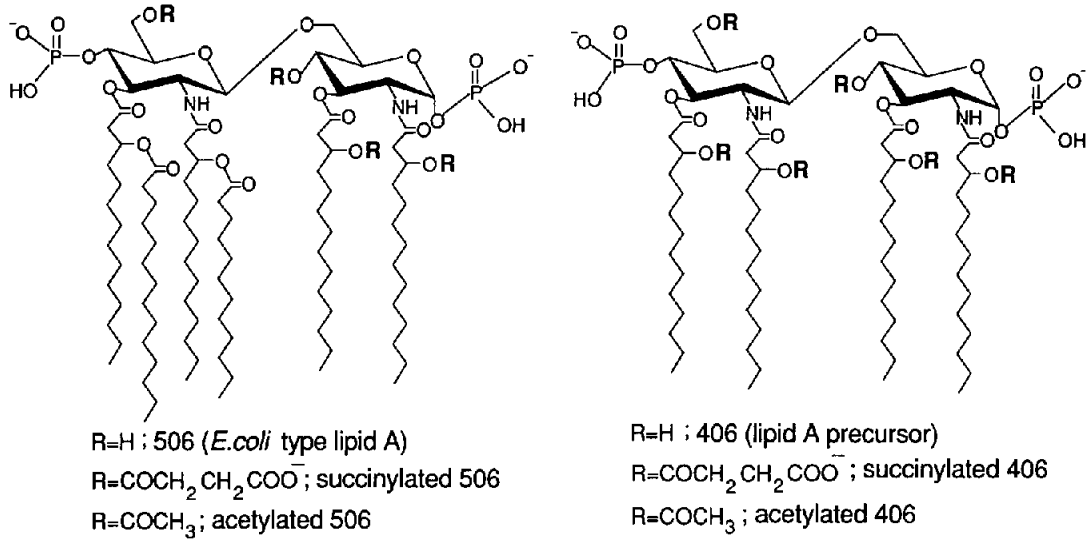

Fig. 1. Structure of the chemically synthesized disaccharide lipid A (506), the precursor (406), and their succinylated and acetylated derivatives. Synthetic lipid A analogue 406 consists of 1,4′-bisphosphorylated glucosamine disaccharide substituted by 4 moles of *R*-3-hydroxy myristic acid at positions 2, 2′, 3, and 3′, and is identical to disaccharide lipid A precursor lipid IV_A or precursor Ia. It lacks 2 acyloxyacylated fatty acids of 506 (complete lipid A of *E. coli*), but is known to express adequate endotoxic activity in animals.

triplicate determinations. Standard errors were less than 10% in the mitogenicity assay and are not indicated in the figures.

2.4.2. Inhibition of mitogenicity. The chemically modified preparations, diluted reciprocally with pyrogen-free water, were added to the assay system together with mitogen in different ratios. The degree of reduction in [^{3}H]thymidine incorporation was measured and compared with a control containing mitogen alone. Inhibition is expressed as the percent mitogenicity calculated thus; cpm of mitogen plus antagonist − cpm of background/cpm of mitogen − cpm of background.

2.4.3. Induction of TNFα release from mouse peritoneal macrophages. Mouse peritoneal macrophages (2×10^6 cells/ml of Iscove's medium) obtained from BALB/C mice (6–10-weeks-old) [19] were incubated with test sample at 37°C for 6 h with 5% CO_2. The TNFα produced in the supernatant was determined by L929 cytotoxicity assay [13]. The results are expressed as means of triplicate determinations.

2.4.4. Lethal toxicity test. The lethality test was performed according to the method described by Galanos et al. [20], using female more than 10-week-old C57BL/6 mice obtained from Nihon SLC (Hamamatsu, Japan). Test samples in 0.1 ml of pyrogen-free water (Hikari Seiyaku Co. Ltd., Tokyo) were injected intravenously immediately after intraperitoneal administration of 12 mg of D-galactosamine (Sigma, St. Louis) in 0.5 ml of pyrogen-free PBS.

3. Results

3.1. Succinylation and acetylation of 406

Mass spectrometry of 406 after succinylation and acetylation showed that five-to-six molecules of succinic residues were equivalently substituted for the 6 free hydroxyl groups of 406, while 6 acetyl residues were efficiently substituted in the precursor. No apparent degradation products, dephosphorylated or deacylated forms, were observed in the spectra of either sample, indicating that no degradation, such as dephosphorylation or deacylation, occurs during the course of chemical modification. The number of succinic residues introduced into 406 was estimated by measuring free succinic acid after hydrolysis of the sample with 4 N HCl using glutaric acid as the internal standard. Succinylation resulted in the introduction of 5.6 mol of succinic residues into the lipid A precursor, providing quantitative confirmation of the results of mass spectrometry.

3.2. Mitogenicity of 406 and its succinylated and acetylated derivatives

The mitogenic activity of lipid A precursor 406 and its succinylated and acetylated derivatives was tested on murine splenic cells. As shown in Fig. 2, 406 exhibited greater mitogenicity in the cells of endotoxin responsive BALB/C mice at both the minimum stimulation dose and the maximum stimulation

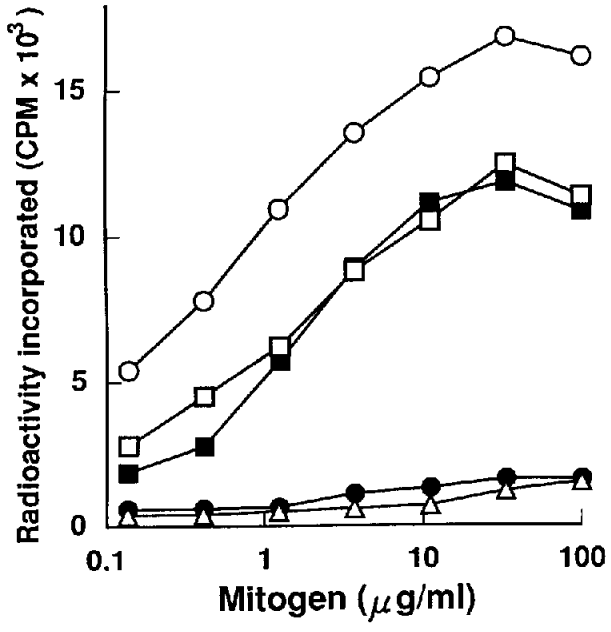

Fig. 2. Mitogenic responses of murine spleen cells to *S. abortus equi* LPS, 506, 406, and its derivatives. Murine spleen cels, 8×10^5 cells/well, were cultured with various concentrations of test samples for 48 h. Radioactivity ([^{3}H]thymidine) incorporated during an additional 24 h of culture was measured. The results are expressed as mean counts per minute in triplicate experiments. The background counts per minute with no lipid A was 645 ± 45 (mean ± S.D.). Symbols: ■, *S. abortus equi* LPS; □, 506; ○, 406; ●, succinylated 406; △, acetylated 406.

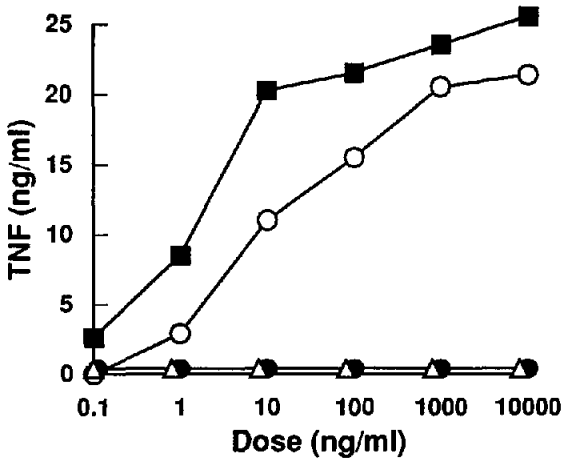

Fig. 3. Induction of TNFα release from murine peritoneal macrophages by LPS, 406 and its derivatives. Murine peritoneal macrophages, 2×10^6 cells/ml, were cultured with various concentrations of test samples for 6 h and the supernatants were assayed for TNFα. The results are expressed as means in triplicate experiments. Symbols: ■, *S. abortus equi* LPS; ○, 406; ●, succinylated 406; △, acetylated 406.

index than LPS from *S. abortus equi* or synthetic complete lipid A (*E. coli* type; 506). Activation occurred at even less than 10 ng/ml, and the maximum cpm incorporated was 17,000, while *S. abortus equi* LPS or 506 stimulates cells at more than 100 ng/ml, and the maximum radioactivity incorporated was 12,000 cpm. The activity of 406 was completely lost both after succinylation and acetylation. No significant mitogenicity was detected with either succinylated or acetylated 406, even at 100 μg/ml. Neither LPS nor 406 stimulated spleen cells from endotoxin non-responsive C3H/HeJ mice (data not shown).

3.3. Induction of TNFα release by 406 and its succinylated and acetylated derivatives

TNFα released into the medium was estimated by cytotoxicity against actinomycin D-sensitized L929 murine fibroblasts. As shown in Fig. 3, murine peritoneal macrophages started to secrete TNFα at a concentration of 1 ng/ml of 406. The production of TNFα by macrophages increased dose-dependently, and maximum TNFα production, 22 ng/ml was observed at the highest concentration tested, 10 μg/ml. Its activity was a little less than that of *S. abortus equi* LPS, which stimulates cells at 0.1 ng/ml and induces 25 ng/ml of TNFα at a concentration of 10 μg/ml. The activity of 406 was lost, however, by introducing succinyl or acetyl residues into the hydroxyl groups. No stimulation was observed even with 100 μg/ml of succinylated 406 or acetylated 406, indicating that its activity had been reduced more than 10^5-fold.

No induction of TNFα release by either LPS or 406 was observed when peritoneal macrophages from C4H/HeJ mice were used.

In order to confirm whether the cytotoxic activity against L929 cells was due to TNFα, aliquots of the supernatants from macrophage culture were incubated for 12 h with polyclonal rabbit antiserum to TNFα using non-specific IgG as the control. The polyclonal antibody to TNFα completely abolished the cytotoxicity of the supernatants stimulated with either LPS or 406.

3.4. Lethality of 406 and its succinylated and acetylated derivatives

The lethal toxicity of the samples was tested using galactosamine-sensitized mice. The results are shown in Table 1. In this system, standard LPS from *S. abortus equi* and synthetic complete lipid A 506 exhibited 100% lethality at 10 ng/mouse and moderate lethality was observed at 1 ng/mouse. Lipid A precursor 406 was lethal at 10 ng/mouse, and at 100 ng/mouse it was 100% lethal. On the other hand, lethality was expressed by neither succinylated nor acetylated 406, even at the maximum dose tested, 100 μg/mouse.

3.5. Inhibition of endotoxic mitogenicity by succinylated and acetylated 406

Since succinylated and acetylated 406 derivatives failed to activate mouse spleen cells, their inhibitory effects on the mitogenicity of *S. abortus equi* LPS were tested by adding them to spleen cell culture togehter with the mitogen. Fig. 4 shows the results of the antagonistic effects of succinylated and acetylated 406 on the mitogenicity of LPS. Dose-dependent inhibition by succinylated 406 was observed over the 1–100 μg/ml range of LPS tested. Significant inhibitory effects were observed even at 10-fold less succinylated 406 than LPS at all stimulation doses, and more than 90% inhibition was possible with 1–10-fold excesses of succinylated 406. The potency of inhibition of acetylated 406 was moderate and about 10 times less than that of succinylated 406 (Fig. 4). Similar inhibition was observed when LPS from *E. coli* or *S. minnesota* R595 (Re type) was used as the agonist. On the other hand, these chemically modified 406 species did not inhibit the mitogenicity of concanavalin A even at 100 μg/ml.

Table 1
406 lost the lethal toxicity on galactosamine-sensitized mice after succinylation or acetylation

	Dose (μg/mouse)					
	0.001	0.01	0.1	1	10	100
	Dead/total					
406		3/10	6/6	6/6		
Succinylated 406				0/3	0/6	0/3
Acetylated 406				0/3	0/6	0/3
LPS from *S. abortus equi*	3/8	6/6				
506	5/15	6/6				

The 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 phosphate-buffered saline.

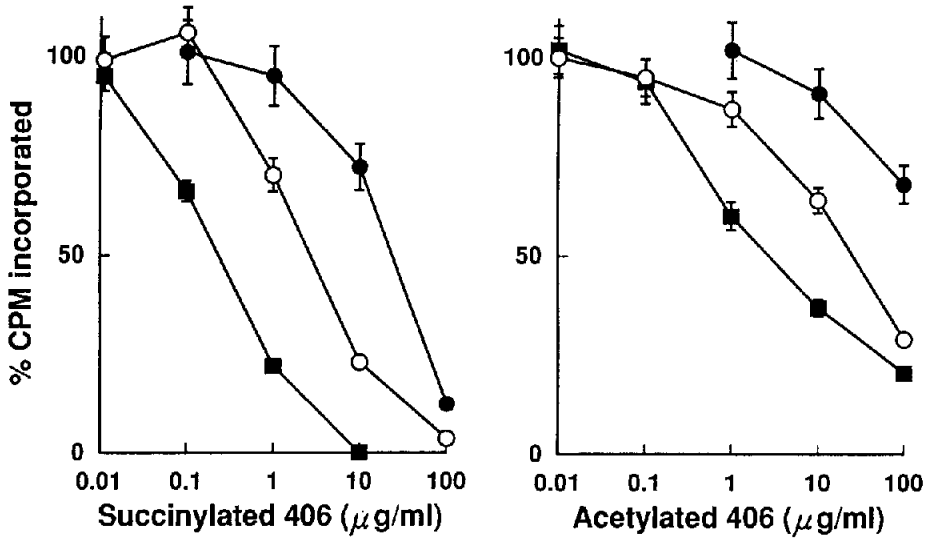

Fig. 4. Inhibition of the splenocyte mitogenic activity of LPS by succinylated and acetylated 406. Reciprocally diluted antagonist in 10 μl of water was added to the splenocytes culture, followed immediately by the addition of 10 μl of various concentrations of *S. abortus equi* LPS and the mitogenicity was tested. Inhibition was expressed as the mean percent mitogenicity ± S.D. of triplicate determinations, taking the incorporation of radioactivity in response to the corresponding concentrations of mitogen alone as 100% and background incorporation as 0%. Concentrations of LPS: ●, 100 μg/ml; ○, 10 μg/ml; ■, 1 μg/ml.

4. Discussion

Recently several nontoxic lipid A structures have been reported [8–10,21]. Such a non-toxic lipid A structure not only gives us more detailed information regarding the structure–activity relationship of lipid A, but also may be used as a potent inhibitor of the toxic action of endotoxin. Since antagonism against endotoxin action is generally observed with the substances which chemically resemble the active lipid A but do not express endotoxicity [22–26], chemical modification of active lipid A is one of the most promising strategies for producing such non-toxic antagonists. My colleague and I found previously that lipid A from *S. abortus equi* loses its mitogenicity for murine spleen cells by the introduction of succinic 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 [27]. The results 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 to an inactive form and into an antagonist of endotoxin. To verify the hypothesis, I expanded my observations by using chemically synthesized pure lipid A analogues. Among the analogues tested, complete lipid A preparations (*E. coli* and *Salmonella* type) were found, unexpectedly, to retain endotoxic activity fully after succinylation [13], indicating that the hydroxyl groups in these complete lipid A preparations are not required to exist in free form for endotoxic activity. In this study I have shown that, in contrast, the disaccharide precursor 406, a partial structure of complete lipid A, loses all of its biological activity upon chemical modification and changes to an antagonist of endotoxin. Introduction of anionic and hydrophilic residues, such as succinic acid, may cause a drastic conformational change in lipid A in water, and hence may lead to the loss of endotoxic activity. Interestingly, however, introduction of non-anionic and hydrophobic acetyl residues into the hydroxyl groups also inactivated the endotoxicity of 406. And both the succinylated and acetylated 406 displayed inhibitory activity on endotoxic mitogenicity. These findings suggest that, regardless of the nature of the substituent, substitution of the hydroxyl groups of 406 is important in both changing the endotoxically active lipid A structure to an inactive form and to an antagonist of endotoxin, i.e. some of the free hydroxyl groups of 406 may be essential to the manifestation of its endotoxic activity. The difference in the chemical structure between the succinylated form of *E. coli* type lipid A and 406 is only the substitution of two hydroxyl residues of 3-hydroxy myristic acid of the non-reducing glucosamine (Fig. 1). This strongly suggests the importance of the substitution of the hydroxyl residues of these positions for the activity and for the transformation to the antagonistic structure. Considering that the hydroxyl residues of these positions are substituted by fatty acids in the complete and active lipid A preparations, the interpretation is not simple. The length of the substituent may also contribute to the activity. Anyway, the dramatic loss of the endotoxic activity of 406 after the substitution indicates that the substituents in these positions apparently modulate the endotoxic activity.

In contrast to 406, neither succinylated nor acetylated 406 exhibited any lethal toxicity when injected intravenously into mice even at the highest dose tested, 100 μg per mouse. For this reason, the present antagonists of endotoxin may be hopeful candidates of application to chemotherapy.

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