Biological Activities of Chemically Synthesized Derivatives of Lipid A: Tetraacetyl-monosaccharides Linked to 2,3-Acyloxyacylglucosamine-4-phosphate

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The mitogenicity and lethal toxicity of chemically synthesized lipid A analogs, in which 2,3-acyloxyacyl-glucosamine-4-phosphate (acyl-GlcN-4P) is linked to a tetraacetyl (Ac_4)-monosaccharide, *i.e.*, Ac_4-glucose (A-211), Ac_4-mannose (A-212), Ac_4-galactose (A-213) or Ac_4-glucosamine (A-214), were compared with those of tetraacetyl-3-deoxy-D-*manno*-2-octulosonic acid (Ac_4-KDO) linked to acyl-GlcN-4P (A-203). All the compounds were capable of increasing incorporation of ^{3}H-thymidine into splenocytes of C57BL/6 mice at doses of 50 and 100 μg/ml, but the mitogenic activity of A-203 at these doses seems to be stronger than those of the analogs. Intravenous injection of A-203, A-211, and A-213 did not exhibit lethal toxicity even at a high dose (50 μg/mouse) in C57BL/6 mice loaded with D-galactosamine hydrochloride. However, A-212 and A-214 showed lethality at the doses of 10 and 50 μg/mouse, respectively. The findings indicate that the biological activity of these compounds is affected by the kind of monosaccharide linked to acyl-GlcN-4P.

Keywords synthetic lipid A analog; mitogenicity; lethal toxicity; lipopolysaccharide

Lipid A is known to be responsible for various biological activities of lipopolysaccharide (LPS) of gram-negative bacteria.[1] Various monosaccharide analogs of lipid A have been synthesized and these compounds, as well as synthetic glucosamine disaccharide analogs of lipid A,[2] showed many of the biological activities of LPS.[3]

Chemical analyses revealed that the C-6 position of the nonreducing glucosamine moiety in lipid A of the Re mutants of *Salmonella* (*S.*) *typhimurium*[4] and *Escherichia coli*[5] is the attachment site to the C-2 position of 3-deoxy-D-*manno*-2-octulosonic acid (KDO), which is well known as the characteristic sugar of the core region in LPS. However, the role(s) of KDO in the various biological activities of LPS is still unclear.

Previously, we reported that compounds consisting of synthetic tetraacetyl(Ac_4)-KDO or KDO linked to 2,3-acyloxyacylglucosamine-4-phosphate (acyl-GlcN-4P) exhibited stronger mitogenicity than the Ac_4-KDO- or KDO-free parent acyl-GlcN-4P compound,[6] but the attachment of Ac_4-KDO or KDO did not affect the antitumor activity against ascites form of Ehrlich carcinoma, the lethal toxicity in galactosamine-loaded mice or the local Shwartzman reaction in the rabbit.[3f,6b,7]

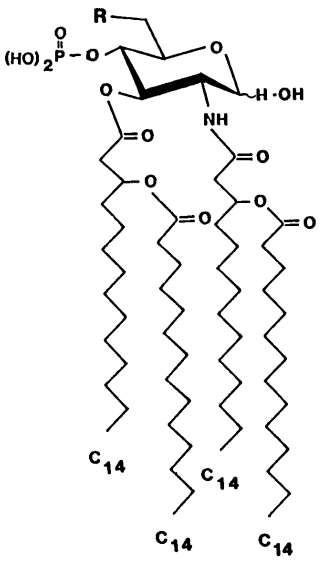
Here, we examined the differences of mitogenicity and lethal toxicity of compounds consisting of synthetic acyl-GlcN-4P linked to various Ac_4-monosaccharides, *i.e.*, Ac_4-glucose (A-211), Ac_4-mannose (A-212), Ac_4-galactose (A-213), Ac_4-glucosamine (A-214) and Ac_4-KDO (A-203).

Materials and Methods

Test Materials Acyl-GlcN-4P carrying Ac_4-KDO (A-203, Fig. 1) was synthesized as described in the previous paper.[8] The syntheses method of compounds A-211—A-214 (Fig. 1) will be published elsewhere. Before experiments each compound was suspended in pyrogen-free saline (Otsuka Pharmaceutical Co., Ltd., Japan) supplemented with 0.1% triethylamine (v/v) and sonicated for 20—30 s. All compounds were slightly turbid in solution and the solubilities appeared to be essentially the same. Reference LPS was isolated from dried cells of *S. typhimurium* LT-2 by the hot phenol–water method[9] and purified by ultracentrifugation (105000 *g*, 1 h).

Animals Male C57BL/6 mice, 7 weeks old, were purchased from the Shizuoka Agricultural Cooperative, Hamamatsu, Japan.

Mitogenicity Test Mitogenicity was tested using murine spleen cells.



compounds

A-203	Ac_4-KDO
A-211	Ac_4-glucose
A-212	Ac_4-mannose
A-213	Ac_4-galactose
A-214	Ac_4-glucosamine

Fig. 1. Structures of Synthetic Lipid A Analogs

Ac_4, tetraacetyl; KDO, 3-deoxy-D-*manno*-2-octulosonic acid.

The splenocytes were suspended in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% fetal bovine serum (GIBCO, Laboratories, Grand Island, N.Y., U.S.A.). One-tenth milliliter (5×10^5 cells) of the cell suspension and 0.1 ml of a suspension of a test compound or reference material were placed in a 96-well microplate (Falcon 3072, Becton Dickinson Labware, Oxnard, CA, U.S.A.). The plate was incubated at 37 °C for 64 h in an atmosphere of 5% CO_2/ 95% air. After addition of 0.25 μCi of ^{3}H-thymidine (Amersham International Ltd., Amersham, England) to each well, the plate was further cultivated for 16 h. Splenocytes were harvested with an automatic cell harvester. Radioactivity uptaken by the cells was measured with a liquid scintillation counter (LSC-661, Aloka Co., Tokyo). Results were expressed as the mean counts per minute (cpm).

Lethal Toxicity Lethality was tested according to the method of Galanos *et al.*[10] In brief, groups of C57BL/6 mice were sensitized by intraperitoneal injection of 640 mg/kg of D-galactosamine hydrochloride in 0.5 ml of saline, followed immediately by intravenous injection of a test compound in 0.2 ml of saline. The mice were observed over a 24 h period, and the number of deaths was determined.

Results and Discussion

The results of the mitogenicity assay are shown in Table

TABLE I. Mitogenic Activity of Synthetic Lipid A Analogs on Cultured Spleen Cells of C57BL/6 Mice *in Vitro*

Material tested	Dose (μg/ml)	^{3}H-Thymidine uptake	
		cpm ± S.D.	S.I.[a]
A-203	100	10069 ± 2240	3.4[b]
	50	9069 ± 1338	3.1[b]
A-211	100	5031 ± 388	1.7[b]
	50	3948 ± 186	1.3[c]
A-212	100	4439 ± 233	1.5[b]
	50	7011 ± 1399	2.4[b]
A-213	100	4352 ± 958	1.5[c]
	50	6171 ± 916	2.1[b]
A-214	100	4240 ± 312	1.4[b]
	50	7265 ± 1596	2.5[b]
S. typhimurium LT-2 LPS	10	15824 ± 3316	5.4[b]
Control (no addition)		2925 ± 152	1.0

Spleen cells (5×10^5) of C57BL/6 mice were cultivated with or without test materials for 48 h and ^{3}H-thymidine (0.25 μCi) was added to each well. After an additional 18 h of culture, the incorporation of ^{3}H-thymidine into cells was measured. *a*) Stimulation index (S.I.) = experimental cpm/control cpm. *b*) Statistically significant compared to control ($p < 0.05$). *c*) Statistically insignificant compared to control ($p > 0.05$).

TABLE II. Lethal Toxicity of Synthetic Lipid A Analogs in Galactosamine-Loaded C57BL/6 Mice

Material tested	No. of deaths/No. of mice tested with following dose (μg)			
	0.1	10	25	50
A-203	n.t.	0/3	0/3	0/3
A-211	n.t.	n.t.	n.t.	0/3
A-212	n.t.	1/3	1/3	2/3
A-213	n.t.	n.t.	n.t.	0/3
A-214	n.t.	0/3	0/3	3/3
S. typhimurium LT-2 LPS	4/4	n.t.	n.t.	n.t.

C57BL/6 mice were treated intraperitoneally with D-galactosamine–HCl (640 mg/kg), and within 30 min, the test preparations were injected intravenously. n.t., not tested.

I. The mitogenic activity of compound A-203 (Ac_4-KDO linked to acyl-GlcN-4P) was observed at doses of 50 and 100 μg/ml, but was weaker than that of *S. typhimurium* LPS. The compounds, A-211—A-214, also possessed mitogenic activity, but appeared to be less potent than A-203. These results were reproducible.

The above results indicate that the mitogenicity of the compound containing Ac_4-KDO is the strongest at the doses of 50 and 100 μg/ml. The reason why Ac_4-KDO resulted in a stronger activity than the other Ac_4-monosaccharides is not clear, but the difference of the potency of these compounds on the mitogenic activity is presumably associated with the difference of chemical structure (for example, KDO and other sugars have 8 carbons and 6 carbons, respectively).

LPS of *S. typhimurium* LT-2 showed lethal toxicity within 6—8 h after treatment at doses of 0.1 μg/mouse (Table II). Compounds A-203, A-211 and A-213 did not cause any death at a dose of 50 μg/mouse. In contrast, the death of all mice was caused by A-214 at 50 μg/mouse. Furthermore, A-212 was toxic to one out of 3 mice at doses of 10 and 25 μg/mouse.

It is of interest that Ac_4-mannose (A-212) or Ac_4-glucosamine (A-214) linked to acyl-GlcN-4P showed stronger lethality than Ac_4-KDO linked to acyl-GlcN-4P. The reason for the differences in the lethality of the compounds remains to be determined.

KDO may be regarded as an essential component of LPS which contributes to the integrity of the bacterial membrane and the unique biological properties of LPS.[11] To determine the role(s) of KDO in the biological activity in more detail, it will be necessary to examine the biological activity of further lipid A analogs.

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