Low Mitogenic Activity of Synthetic Lipid A Analog, 2,3-Acyloxyacylgalactosamine-4-phosphate, in Cultured Murine Splenocytes

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The mitogenicity of chemically synthesized lipid A analogs, 2,3-acyloxyacylglucosamine-4-phosphate (A-103) and 2,3-acyloxyacylgalactosamine-4-phosphate (A-113), was compared. Synthetic lipid A analogs of the disaccharide-type (506), the monosaccharide-type (A-103) suspended in RPMI-1640 medium supplemented with triethylamine, and *Salmonella typhimurium* LT-2 lipopolysaccharide (LPS) were capable of increasing the incorporation of [3 H]thymidine into splenocytes of C57BL/6 mice at various doses ranging from 1.0 to 100 μ g/ml. However, the mitogenic activity of A-113 at these doses was markedly weaker than the activity of the above materials. When the A-103 and A-113 were suspended in RPMI-1640 medium supplemented with ethanol, the mitogenic activity of A-113 also showed lower activity than that of A-103 in the splenocytes of C57BL/6 mice. These findings indicate that the mitogenic activity of synthetic lipid A of the monosaccharide-type is affected by a kind of composed sugar.

Keywords lipid A analog; lipopolysaccharide; mitogenicity

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

Bacterial LPS usually consists of a glucosaminyl- β -D-glucosamine as a backbone sugar of lipid A.¹⁾ The examination of the biological activity of synthetic lipid A analogs of the monosaccharide-type possessing other kinds of sugar is therefore of interest.

Here, we examined the differences of mitogenicity between two synthetic lipid A analogs, 2,3-acyloxyacylglucosamine-4-phosphate (A-103) and 2,3-acyloxyacylgalactosamine-4-phosphate (A-113), in the splenocytes of C57BL/6 mice.

Materials and Methods

Test Materials Compound A-103 was synthesized from D-glucosamine-HCl as described in a previous paper.³⁾ A-113 was synthesized from D-galactosamine-HCl in nine steps, and detailed methods will be published elsewhere. The chemical structures of these compounds are shown in Fig. 1. Each compound was suspended in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 0.1% triethylamine (v/v) or 5% ethanol (v/v) and sonicated for 20–30 s. For use, the stock solutions were serially diluted to the required concentrations with RPMI-1640 medium. The final concentration of triethylamine or ethanol in the tested solutions was less than 0.01% or 0.25%, respectively. Synthetic lipid A (506) of the *Escherichia coli*-type was purchased from Dai-ichi Pure Chemicals Co., Tokyo. The natural LPS of *S. typhimurium* LT-2 was isolated from dried cells by the hot phenol–water method⁴⁾ and purified

by ribonuclease (RNase, Sigma Chemical Co., St. Louis, MO, U.S.A.) and ultracentrifugation (105000 $\times g$, 1 h).

Animals Male C57BL/6 mice, 5 weeks old, were purchased from the Japan SLC Co. (Hamamatsu).

Mitogenicity Test Mitogenicity was tested using murine spleen cells. The splenocytes were suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (Irvine Scientific, Co., CA, 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 materials were placed in a 96-well microplate (Falcon 3072, Becton Dickinson Labware, Oxnard, CA, U.S.A.). The plate was incubated at 37 $^{\circ}$ C for 64 h in an atmosphere of 5% CO₂/95% air. After the addition of 9.25 kBq of [3 H]thymidine (New England Nuclear, Corp., Boston, MA, U.S.A.) to each well, the plate was further cultivated for 16 h. Splenocytes were harvested with an automatic cell harvester. Radioactivity uptake by the cells was measured with a liquid scintillation counter (LSC-3100, Aloka Co., Tokyo). Results were expressed as the mean counts per min (cpm) of triplicate wells per a group of each concentration.

Statistical Analysis The significance of the results was analyzed using the Student's *t* test. Probability values of less than 0.05 were considered significant.

Results and Discussion

The results of the mitogenic assay of the compounds A-103 and A-113, which were suspended in RPMI-1640 medium supplemented with triethylamine, in the splenocytes of C57BL/6 mice are shown in Table I.

The mitogenic activity of compound A-103 was observed at various doses of 1.0 to 100 μ g/ml, but was weaker than that of 506, *S. typhimurium* LPS. The compound, A-113, also possessed weak mitogenic activity at doses of 5, 10 and 25 μ g/ml, but exhibited lower potency than A-103. These results were reproducible.

Next, we added 5% ethanol to a stock solution of A-103 and A-113, and the suspensions were sonicated. As shown in Table II, the mitogenic activity of A-113 was lower than that of A-103 at various doses ranging from 5.0 to 50 μ g/ml. Therefore, under the conditions involving ethanol or triethylamine supplementation, the finding that the galactosamine-4-phosphate (A-113) has lower mitogenicity than the glucosamine-4-phosphate (A-103) was also observed.

The reason compound A-113 resulted in a lower activity than compound A-103 is not clear, but the difference of the potency between the compounds regarding mitogenic

Fig. 1. Chemical Structures of A-103 and A-113

R = C₁₄-O-C₁₄; (R)-3-tetradecanoyloxytetradecanoyl.

TABLE I. Mitogenic Activity of Synthetic Lipid A Analogs in RPMI-1640 Medium Supplemented with Triethylamine on Cultured Splenocytes of C57BL/6 Mice *in Vitro*

Materials tested	Dose ($\mu\text{g/ml}$)	[^3H]Thymidine uptake	
		Mean cpm $\pm$ S.D.	S.I. ^{a)}
A-103	100	13369 $\pm$ 1069	5.9 ^{b)}
	50	19018 $\pm$ 4400	8.4 ^{b)}
	25	15485 $\pm$ 1306	6.8 ^{b)}
	10	18877 $\pm$ 2255	8.3 ^{b)}
	5	14523 $\pm$ 2010	6.4 ^{b)}
	1	10517 $\pm$ 866	4.6 ^{b)}
A-113	100	2032 $\pm$ 191	0.9
	50	3086 $\pm$ 400	1.4
	25	3963 $\pm$ 514	1.7 ^{b)}
	10	4580 $\pm$ 697	2.0 ^{b)}
	5	3963 $\pm$ 571	1.7 ^{b)}
	1	3072 $\pm$ 844	1.4
506	50	39421 $\pm$ 5200	17.4 ^{b)}
	10	40607 $\pm$ 5315	17.9 ^{b)}
<i>S. typhimurium</i>	50	34384 $\pm$ 2041	15.2 ^{b)}
LT-2 LPS	10	47663 $\pm$ 1687	21.0 ^{b)}
Control (no addition)		2268 $\pm$ 156	1.0

a) stimulation index (S.I.) = experimental cpm/control cpm. b) Statistically significant compared to control ($p < 0.05$).

TABLE II. Mitogenic Activity of Synthetic Lipid A Analogs in RPMI-1640 Medium Supplemented with Ethanol on Cultured Splenocytes of C57BL/6 Mice *in Vitro*

Materials tested	Dose ($\mu\text{g/ml}$)	[^3H]Thymidine uptake	
		Mean cpm $\pm$ S.D.	S.I. ^{a)}
A-103	50	13301 $\pm$ 2485	5.1 ^{b)}
	25	12840 $\pm$ 1741	4.9 ^{b)}
	10	10201 $\pm$ 2531	3.9 ^{b)}
	5	4652 $\pm$ 411	1.8 ^{b)}
	1		
A-113	50	6484 $\pm$ 2880	2.5
	25	3124 $\pm$ 576	1.2
	10	1982 $\pm$ 100	0.8
	5	2599 $\pm$ 178	1.0
<i>S. typhimurium</i>	10	56857 $\pm$ 2238	21.6 ^{b)}
LT-2 LPS			
Control (no addition)		2628 $\pm$ 660	1.0

a) See legend a) in Table I. b) Statistically significant compared to control ($p < 0.05$).

activity is presumably associated with the difference in solubility.

Lipid A of bacterial LPS consists of a glucosaminyl- β -D-glucosamine disaccharide as a backbone sugar.¹⁾ However, galactosamine phosphate as a backbone sugar of lipid A has not been found in the LPS of any bacteria. There are many papers regarding LPS containing a novel lipid A structure which is composed mainly of 2,3-diamino-2,3-dideoxy-D-glucose (DGA).⁵⁾ Kasai *et al.* showed that lipid A containing DGA as well as the usual lipid A is able to induce the production of tumor necrotizing factor.⁶⁾ Examination of the biological activity of lipid A analogs possessing other types of sugars as the backbone sugar may be useful for the development of new immunomodulators.

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