Volume 228, number 1, 99-101 FEB 05542 February 1988

Biological activities of chemically synthesized *N*-acetylneuraminic acid-(α2→6) -monosaccharide analogs of lipid A

Tadayori Shimizu, Toshiyuki Masuzawa, Yasutake Yanagihara, Chikako Shimizu[+], Kiyoshi Ikeda[+] and Kazuo Achiwa[+]

Department of Microbiology and [+] *Department of Medicinal Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 2-2-1 Oshika, Shizuoka 422, Japan*

Received 6 November 1987; revised version received 8 December 1987

The mitogenicity and lethal toxicity of chemically synthesized lipid A analogs, in which 2,3-acyloxyacylglucosamine-4-phosphate linked to tetraacetyl-*N*-acetylneuraminic acid (compound A-207) or to *N*-acetylneuraminic acid (compound A-307), were examined. Although the mitogenic activity of the synthetic compounds was weaker than that of bacterial LPS, doses of 10–50 μg/ml of A-207 and 5–10 μg/ml of A-307 were capable of increasing incorporation of [^{3}H]thymidine into cultured spleen cells of C57BL/6 mice. Lethal toxicity of A-207 was observed at 10 μg/mouse in C57BL/6 mice sensitized with D-galactosamine hydrochloride. However, the attachment of tetraacetyl-*N*-acetylneuraminic acid or *N*-acetylneuraminic acid does not appear to enhance the biological activity of acyloxyacylglucosamine-4-phosphate.

Synthetic lipid A analog; *N*-Acetylneuraminic acid; Mitogenic activity; Lethal toxicity

1. INTRODUCTION

Lipid A of bacterial lipopolysaccharide (LPS) is well known to possess many biological activities [1]. Recently, various derivatives of acyloxyacylglycosamine-4-phosphate as monosaccharide analogs of lipid A have been synthesized, and these compounds as well as synthetic glucosamine disaccharide analogs of lipid A [2–5] induced the production of interferon and tumor necrosis factor, and secretion of interleukin I, and exhibited mitogenic activity for B-lymphocytes, antitumor activity, and lethal toxicity, etc. [6–13].

There are many reports that gangliosides play roles in immune responses [14]. *N*-Acetylneuraminic acid-containing gangliosides were found to inhibit the LPS-induced activation of murine B-lymphocytes [15]. The biological activities of monosaccharide analogs of lipid A *N*-acetylneuraminic acid are therefore of interest.

Correspondence address: T. Shimizu, Department of Microbiology, School of Pharmaceutical Sciences, University of Shizuoka, 2-2-1 Oshika, Shizuoka 422, Japan

Here, we examined the mitogenicity and lethal toxicity of chemically synthesized acyloxyacylglucosamine-4-phosphate (Acyl-GlcN-4-P) linked to tetraacetyl-*N*-acetylneuraminic acid (Ac_4-NeuAc, compound A-207) or *N*-acetylneuraminic acid (NeuAc, compound A-307).

2. MATERIALS AND METHODS

The compounds tested in this study, A-207 and A-307, were synthesized as described in [16]; their chemical structures are shown in fig.1. Before experiments, each compound was suspended in pyrogen-free saline supplemented with 0.1% triethylamine (v/v) and sonicated for 20–30 s. Reference LPS was isolated from dried cells of *Salmonella typhimurium* LT-2 by the hot phenol-water method [17] and purified by ultracentrifugation (105 000 × *g*, 1 h).

Mitogenicity was tested using spleen cells of C57BL/6 mice. The splenocytes were suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum. 0.1 ml (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. 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 [^{3}H]thymidine to each well, the plate was further cultivated for 16 h. Splenocytes were harvested with an automatic cell

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

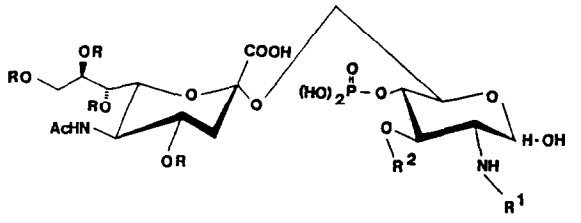

Fig. 1. Structure of synthetic lipid A analogs linked to *N*-acetylneuraminic acid. Ac, acetyl; C_{14}-*O*-C_{14} (*R*)-3-tetradecanoyloxy-tetradecanoyl.

Compounds	R^1 (*N*-)	R^2 (3-*O*-)	*R*
A-207	C_{14}-*O*-(C_{14})	C_{14}-*O*-(C_{14})	Ac
A-307	C_{14}-*O*-(C_{14})	C_{14}-*O*-(C_{14})	H

harvester. Radioactivity taken up by the cells was measured with a liquid scintillation counter. Results were expressed as mean cpm.

Lethal toxicity was tested according to Galanos et al. [18]. In brief, groups of C57BL/6 mice were sensitized by intraperitoneal injection of 640 mg/kg of D-galactosamine hydrochloride in 0.5 ml saline, followed immediately by intravenous injection of a test compound in 0.2 ml saline. The mice were observed over a 24 h period, and the number of deaths recorded.

3. RESULTS AND DISCUSSION

The results of the mitogenic assay of A-207 and A-307 are listed in table 1. Compound A-207 exhibited slight mitogenic activity at a dose of 10 μg/ml or more. On the other hand, the mitogenic activity of A-307 was also observed at doses of 5 and 10 μg/ml but seemed to be weaker than that of A-207. However, the attachment of Ac_4-NeuAc or NeuAc does not appear to enhance the activity of acyl-Glcn-4-P.

Previously, we reported that the mitogenic activity of acyl-GlcN-4-P linked to tetra-acetyl-3-deoxy-D-manno-2-octulosonic acid (Ac_4-KDO) was stronger than that of the original acyl-GlcN-4-P derivatives [19]. There are some structural similarities between *N*-acetylneuraminic acid and KDO, since they have a ketosidic linkage and an acidic group in the pyranosyl skeleton. An explanation for the difference in mitogenic activities between the derivatives of Ac_4-NeuAc and Ac_4-KDO awaits discovery.

Table 1

Mitogenic activity of synthetic lipid A analogs

Preparations	Dose (μg/ml)	[^{3}H]TdR uptake (cpm ± SD)	S.I.
Expt 1			
A-207	50	3682 ± 860	2.2[a]
	25	3791 ± 524	2.3[a]
	10	2968 ± 470	1.8[a]
	1	2556 ± 173	1.5[a]
	0.5	2323 ± 34	1.4
S. typhimurium LT-2 LPS	10	13090 ± 4080	7.8
Control (no addition)		1668 ± 379	1.0
Expt 2			
A-307	50	2069 ± 44	0.9
	25	2669 ± 65	1.2
	10	3795 ± 58	1.7[a]
	5	3829 ± 249	1.7[a]
	1	3277 ± 115	1.4
S. typhimurium LT-2 LPS	10	30680 ± 2269	13.3
Control (no addition)		2311 ± 143	1.0

[a] $P<0.05$

S.I. (stimulation index) = experimental cpm/control cpm

LPS of *S. typhimurium* LT-2 showed lethal toxicity within 6–8 h after treatment at doses of 0.1 and 1.0 μg/mouse (table 2). Compound A-207 was toxic to 3 out of 4 mice at a dose of 10 μg/mouse, and all mice were dead at doses of 25 and 50 μg/mouse. The lethal toxicity of A-207 is considered to be almost the same as that of Ac_4-NeuAc free compounds [9, 10].

A-207 could not induce the local Shwartzman reaction at doses of 40 and 80 μg/site in rabbit (not shown). From this finding and other results [2–5], it is assumed that the induction of the Shwartzman reaction requires the presence of glucosamine disaccharide in lipid A.

This is the first report on the mitogenic activity

Table 2

Lethal toxicity of synthetic lipid A analog (A-207) in galactosamine-sensitized C57BL/6 mice

Preparations	No. of deaths/no. of mice tested at indicated dose (μg)					
	0.1	1.0	5	10	25	50
A-207	ND[a]	0/4	0/4	3/4	4/4	4/4
S. typhimurium LT-2 LPS	3/3	4/4	ND	ND	ND	ND

[a] ND, not done

and lethal toxicity of acyl-GlcN-4-P linked to *N*-acetylneuraminic acid or tetraacetyl-*N*-acetylneuraminic acid. Our results indicate that the attachment of *N*-acetylneuraminic acid does not enhance the biological activities of acyl-GlcN-4-P. However, the possibility that the compounds may exhibit some biological activities in other experiments cannot be ruled out. Further chemical modification of lipid A analogs may yield new biological response modifiers.

Acknowledgements: This work was supported in part by a Grant-in Aid from the Tokyo Biochemical Research Foundation. We thank Misses A. Washizu and T. Maki for technical assistance.

REFERENCES

[1] Lüderitz, O., Galanos, C., Lehmann, V., Mayer, H., Rietschel, E.T. and Weckesser, J. (1978) Naturwissenschaften 65, 578–585.

[2] Kotani, S., Takada, H., Tsujimoto, M., Ogawa, T., Takahashi, I., Ikeda, T., Otsuka, K., Shimauchi, H., Kasai, N., Mashimo, J., Nagao, S., Tanaka, A., Tanaka, S., Harada, K., Nagaki, K., Kitamura, H., Shiba, T., Kusumoto, S., Imoto, M. and Yoshimura, H. (1985) Infect. Immun. 49, 225–237.

[3] Homma, J.Y., Matsuura, M., Kanegasaki, S., Kawakubo, Y., Kojima, Y., Shibukawa, N., Kumazawa, Y., Yamamoto, A., Tanamoto, K., Yasuda, T., Imoto, M., Yoshimura, H., Kusumoto, S. and Shiba, T. (1985) J. Biochem. (Tokyo) 98, 395–406.

[4] Kanegasaki, S., Tanamoto, K., Yasuda, T., Homma, J.Y., Matssura, M., Nakatsuka, M., Kumazawa, Y., Yamamoto, A., Shiba, T., Kusumoto, S., Imoto, M., Yoshimura, H. and Shimamoto, T. (1986) J. Biochem. (Tokyo) 99, 1203–1210.

[5] Shimizu, T., Akiyama, S., Masuzawa, T., Yanagihara, Y., Ikeda, K., Takahashi, T., Kondo, H. and Achiwa, K. (1987) Microbiol. Immunol. 31, 381–386.

[6] Charon, D., Chaby, R., Malinvaud, A., Mondange, M. and Szabó, L. (1984) Biochemistry 24, 2736–2742.

[7] Matsuura, M., Kojima, Y., Homma, J.Y. Kubota, Y., Yamamoto, A., Kiso, M. and Hasegawa, A. (1984) FEBS Lett. 167, 226–230.

[8] Kumazawa, Y., Matsuura, M., Homma, J.Y. Nakatsuru, Y., Kiso, M. and Hasegawa, A. (1985) Eur. J. Immunol. 15, 199–201.

[9] Shimizu, T., Akiyama, S., Masuzawa, T., Yanagihara, Y., Nakamoto, S., Takahashi, T., Ikeda, K. and Achiwa, K. Chem. Pharm. Bull. 33, 4621–4624.

[10] Shimizu, T., Akiyama, S., Masuzawa, T., Yanagihara, Y., Nakamoto, S., Takahashi, T., Ikeda, K. and Achiwa, K. (1986) Chem. Pharm. Bull. 34, 5169–5175.

[11] Sayer, T.J., Macher, I., Chung, J. and Kugler, E. (1987) J. Immunol. 138, 2935–2940.

[12] Shimizu, T., Akiyama, S., Masuzawa, T., Yanagihara, Y., Nakamoto, S. and Achiwa, K. (1987) Infect. Immun. 55, 2287–2289.

[13] Takahashi, I., Kotani, S., Takada, H., Tsujimoto, M., Ogawa, T., Shiba, T., Kusumoto, S., Yamamoto, M., Hasegawa, A., Kiso, M., Nishijima, M., Amano, F., Akamatsu, Y., Harada, K., Tanaka, S., Okamura, H. and Tamura, T. (1987) Infect. Immun. 55, 57–68.

[14] Dyatlovitskaya, E.V. and Bergelson, L.D. (1987) Biochim. Biophys. Acta 907, 125–143.

[15] Ryan, J.L. and Shinitzky, M. (1979) Eur. J. Immunol. 9, 171–175.

[16] Shimizu, C., Ikeda, K. and Achiwa, K. (1987) Chem. Pharm. Bull., (in press).

[17] Westphal, O., Lüderitz, O. and Bister, F. (1953) Z. Naturforsch, 7B, 148–155.

[18] Galanos, C., Freudenberg, M.A. and Reutter, W. (1979) Proc. Natl. Acad. Sci. USA 76, 5939–5943.

[19] Shimizu, T., Akiyama, S., Masuzawa, T., Yanagihara, Y., Nakamoto, S. and Achiwa, K. (1987) Chem. Pharm. Bull. 35, 873–976.