Biological activities of chemically synthesized analogues of the nonreducing sugar moiety of lipid A

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Biological activities of five synthetic lipid A analogues (D-glucosamine derivatives) were examined to elucidate the structure required for expression of the biological activities of endotoxin. Proclotting enzyme of horseshoe crab activation, interferon-inducing and tumor necrosis factor-inducing activities were significantly expressed by an analogue which possesses 4-*O*-phosphoryl, 3-*O*-tetradecanoyl and *N*-tetradecanoyloxytetradecanoyl groups. The results obtained with different analogues show that the 4-*O*-phosphoryl and *N*-tetradecanoyloxytetradecanoyl groups are important for expression of the above activities. The effect of 6-*O*-acylation in preventing the expression of these biological activities is also suggested. Pyrogenic activity was not detected in any of the compounds tested.

Synthetic lipid A analogue *Limulus test* *Interferon-induction* *Tumor necrosis factor induction* *Pyrogenicity* *Lipid A*

1. INTRODUCTION

Most of the biological activities of endotoxin have been shown to be expressed by its lipid A moiety, and the chemical structure of lipid A has been proposed based on detailed investigations of *Salmonella* lipid A [1,2]. Chemical synthesis of the proposed structure has been attempted [3,4]. As a consequence, various synthetic lipid A analogues have become available and were used to elucidate the relationship between chemical structures and biological activities of lipid A.

The authors and others have investigated the biological activities of synthetic lipid A analogues and confirmed that some analogues of D-glucosamine disaccharide derivatives exhibited certain biological activities of the endotoxin [5–7], but not all endotoxic activities. At the same time, the importance of both phosphoryl groups and amino- and ester-linked acyl groups for expression of the biological activities was noted.

A series of chemically synthesized derivatives of D-glucosamine, which are structurally related to the nonreducing sugar moiety of a newly proposed structure of lipid A [8,9], were examined here for proclotting enzyme of horseshoe crab activation, pyrogenicity and interferon- and TNF-induction.

Abbreviations: TNF, tumor necrosis factor; C_{14}, tetradecanoyl; C_{14}-O-(C_{14}), (R)-3-tetradecanoyloxytetradecanoyl; C_{14}-OH, (R)-3-hydroxytetradecanoyl; C_{12}-OH, (R)-3-hydroxydodecanoyl

2. MATERIALS AND METHODS

The structures of synthetic lipid A analogues [10,11] used are shown in fig.1. These analogues are the derivatives of *N*-acyl-3-*O*-tetradecanoyl-D-glucosamine with or without 4-*O*-phosphoryl or 6-*O*-tetradecanoyl groups. *N*-Acyl groups are C_{14}, C_{14}-O-(C_{14}), C_{12}-OH or C_4-OH groups.

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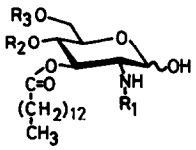

Fig. 1. Structures of synthetic lipid A analogues. P, phosphoryl; C_{14}, tetradecanoyl; C_{12}-OH, (R)-3-hydroxydodecanoyl; C_{14}-OH, (R)-3-hydroxytetradecanoyl; C_{14}-O-(C_{14}), (R)-3-tetradecanoyloxytetradecanoyl.

Compound no.	Substituent		
	R_1 (*N*-)	R_2 (4-*O*-)	R_3 (6-*O*-)
1	C_{14}-O-(C_{14})	H	H
2	C_{14}	P	H
3	C_{14}-O-(C_{14})	P	H
4	C_{12}-OH	P	C_{14}
5	C_{14}-OH	P	C_{14}

The synthetic lipid A analogues and natural lipid A were solubilized with triethylamine and bovine serum albumin prior to biological testing, and recovery of each preparation was determined by gas liquid chromatography as in [5].

Proclotting enzyme of horseshoe crab activation, interferon-induction and pyrogenicity were assayed as in [5].

TNF-inducing activity [12] was assayed as follows: 6-week-old ICR female mice were injected intraperitoneally with 1.5 mg of formalin-killed *Propionibacterium acnes*. After 11 days, 10 μg of test sample was injected intravenously and blood was collected 1.5 h later from the retroorbital plexus. Serum was separated by centrifugation and incubated at 56°C for 30 min. TNF activity in the serum was assayed by measuring growth inhibitory and cytocidal activities on L-929 cells. For growth inhibitory assay, 100-fold diluted serum and [^{3}H]thymidine were added to 1×10^4 L-929 cells in RPMI-1640 medium containing 10% fetal calf serum (200 μl in total volume/well of a 96 well-plate) and the mixture was incubated in 5% CO_2–air at 37°C for 48 h. The viable cells were harvested with 0.25% trypsin and 0.1% EDTA using a multiple cell harvester. The amount of [^{3}H]thymidine incorporated into the cells was measured by a liquid scintillation spectrometer. For cytocidel assay, 40-fold diluted serum was used and assayed as in [13].

3. RESULTS

After solubilization, recovery of compound 1 was about 80%. Those of the other 4 compounds were more than 95%. Turbidity at 660 nm of each preparation (200 μg/ml) of the compounds 1, 2, 3, 4 and 5 was 0.275, 0.010, 0.001, 0.098 and 0.129, respectively. As for compounds 1–3, 3 preparations of each compound were made and similar results were obtained.

As shown in table 1, compounds 2 and 3 expressed strong activity of proclotting enzyme of

Table 1

Proclotting enzyme of horseshoe crab activation activity of the synthetic lipid A analogues

Compound no.	Substituent			LAL[a] gelation at the concentration (μg/ml) of					
	N- (R_1)	4-*O*- (R_2)	6-*O*- (R_3)	10	1.0	10^{-1}	10^{-2}	10^{-3}	10^{-4}
1	C_{14}-O-(C_{14})	H	H	+	−	−			
2	C_{14}	P	H	+	+	+	+	±	−
3	C_{14}-O-(C_{14})	P	H	+	+	+	+	+	±
4	C_{12}-OH	P	C_{14}	+	−	−			
5	C_{14}-OH	P	C_{14}	+	−	−			
Control	None			+	−	−			
	Lipid A					+	+	+	±

[a] LAL: *Limulus* amoebocyte lysate

Table 2

Interferon-inducing activity of synthetic lipid A analogues

Compound no.	Substituent			Experiment no.	Units of interferon induced at the concentration (μg/ml) of					
	N- (R_1)	4-*O*- (R_2)	6-*O*- (R_3)		10	1.0	10^{-1}	10^{-2}	10^{-3}	10^{-4}
1	C_{14}-O-(C_{14})	H	H	1	12	<10	<10			
				2	<10	<10	<10			
				3	<10	<10	<10			
2	C_{14}	P	H	1	<10	<10	<10			
				2	<10	<10	<10			
				3	<10	<10	<10			
3	C_{14}-O-(C_{14})	P	H	1	213	97	<10			
				2	90	28	<10			
				3	190	113	19			
4	C_{12}-OH	P	C_{14}	4	<10	<10	<10			
5	C_{14}-OH	P	C_{14}	4	<10	<10	<10			
Control	None			1	<10	<10				
				2	<10	<10				
				3	<10	<10				
				4	<10	<10				
	Lipid A			1			427	22	<10	<10
				2			200	130	48	<10
				3			605	390	26	<10
				4			154	23	<10	<10

horseshoe crab activation: the activity of compound 3 was the same as that of natural lipid A and that of compound 2 was one order less. No such activity was detected in the other 3 compounds. Three preparations each of compounds 1, 2 and 3 were subjected to this assay and similar results were obtained.

Interferon-inducing activity was found clearly only in compound 3, as shown in table 2. The activity is fairly strong, but the minimal effective concentration was 2–3 orders of magnitude higher than that of natural lipid A.

Pyrogenicity of compounds 1–3 was tested using rabbits. No activity was detected in any of the 3 compounds at 30 μg/kg, the highest dose examined.

TNF-inducing activity of compounds 1–3 was also tested. As shown in table 3, compound 3 expressed relatively strong activity, compound 2 showed weak but significant activity, and compound 1 showed no activity at all.

4. DISCUSSION

Three out of 4 biological activities described above were significantly expressed by one of the synthetic lipid A analogues (compound 3). This compound is a derivative of 3-*O*-tetradecanoyl-D-glucosamine possessing 4-*O*-phosphoryl and *N*-tetradecanoyloxytetradecanoyl groups. Compound 1, which is a nonphosphorylated derivative of compound 3, showed none of those activities. This clearly indicates the importance of the 4-*O*-phosphoryl group for expression of these activities.

Comparing the activities of compounds 2 and 3, the activity of proclotting enzyme of horseshoe crab activation of both compounds was as strong as that of natural lipid A, although that of compound 2 was one order of magnitude less than that of compound 3. As for TNF-inducing activity, compound 3 expressed relatively strong activity, but the activity in compound 2 was weak. Inter-

Table 3

Tumor necrosis factor (TNF)-inducing activity of synthetic lipid A analogues

Compound no.	Substituent			Activity of induced TNF on L-929 cells			
	N- (R_1)	4-*O*- (R_2)	6-*O*- (R_3)	Growth inhibition		Cytocidal effect	
				([^{3}H]TdR uptake)[a]	(%)[b]	(dead/viable)[c]	(%)[d]
1	C_{14}-O-(C_{14})	H	H	31 767 ± 1 039	5.5	7/140	4.8
2	C_{14}	P	H	24 968 ± 1 007	26.0*	22/100	18.0*
3	C_{14}-O-(C_{14})	P	H	6 513 ± 763	80.4**	69/22	77.5**
Control	None			32 999 ± 1 124	1.9	6/160	3.6
	Lipid A			1 457 ± 273	95.7**	60/2	96.7**

[a] Mean cpm ± SE in triplicate; [b] % Growth inhibition = [1 − (cpm in a test serum)/(cpm in a control without serum)] × 100; [c] × 10^4 cells/ml, viable and dead cells were determined using phase microscopy; [d] % Cytocidal effect = (number of dead cells/number of total cells) × 100

* $P < 0.01$, ** $P < 0.001$

feron-inducing activity was clearly shown by compound 3 but the activity in compound 2 was undetectable. Compound 2 is a derivative of compound 3 which possesses the C_{14} group as the *N*-acyl group instead of the C_{14}-O-(C_{14}) group in compound 3. The results indicate that not only the 4-*O*-phosphoryl group but also the *N*-acyl group contribute largely to the expression of TNF- and interferon-induction activities: the C_{14}-O-(C_{14}) group is a much more effective *N*-acyl substituent than the C_{14} group.

Compounds 4 and 5 are derivatives of compound 3 which possess additional 6-*O*-tetradecanoyl groups and altered *N*-acyl groups such as a C_{12}-OH group (compound 4) or a C_{14}-OH group (compound 5). These compounds expressed neither proclotting enzyme of horseshoe crab activation nor interferon-inducing activities even though they possess the 4-*O*-phosphoryl group. In our previous study [5,6] it was shown that not only the C_{14}-O-(C_{14}) group but the C_{14}-OH group is also a more effective substituent than the C_{14} group as the *N*-acyl group of glucosamine disaccharide derivatives of synthetic lipid A analogues. Judging from these results it is suggested that the 6-*O*-acylation prevents or reduces the expression of these activities.

As described above, all the biological activities tested are significantly expressed by compound 3. This compound fulfills nearly completely the structure requirement of the nonreducing sugar moiety of the revised structure of lipid A which has been proposed recently [8,9]. This result is a strong indication that several biological activities of the endotoxin can be clearly expressed solely by the structure of the nonreducing sugar moiety of lipid A.

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REFERENCES

[1] Galanos, C., Lüderitz, O., Rietschel, E.T. and Westphal, O. (1977) Int. Rev. Biochem. 14, 239–335.

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

[3] Inage, M., Chaki, H., Kusumoto, S. and Shiba, T. (1980) Tetrahedron Lett. 21, 3889–3892.

[4] Kiso, M., Nishiguchi, H., Murase, S. and Hasegawa, A. (1981) Carbohydr. Res. 88, C5–C9.

[5] Matsuura, M., Kojima, Y., Homma, J.Y., Kubota, Y., Shibukawa, N., Shibata, M., Inage, M., Kusumoto, S. and Shiba, T. (1983) Eur. J. Biochem., in press.

[6] Kumazawa, Y., Matsuura, M., Nakatsuru-Watanabe, Y., Fukumoto, M., Nishimura, C., Homma, J.Y., Inage, M., Kusumoto, S. and Shiba, T. (1983) Eur. J. Immunol., in press.

[7] Yasuda, T., Kanegasaki, S., Tsumita, T., Tadakuma, T., Homma, J.Y., Inage, M., Kusumoto, S. and Shiba, T. (1982) Eur. J. Biochem. 124, 405–407.
[8] Takayama, K., Qureshi, N., Mascagni, P., Nashed, M.A., Anderson, L. and Raetz, C.R.H. (1983) J. Biol. Chem. 258, 7379–7385.
[9] Imoto, M., Kusumoto, S., Shiba, T., Naoki, H., Iwashita, T., Rietschel, E.T., Wollenweber, H.-W., Galanos, C. and Lüderitz, O. (1983) Tetrahedron Lett. 24, 4017–4020.
[10] Kiso, M., Ishida, H. and Hasegawa, A. (1984) Agric. Biol. Chem. 48, 251–252.
[11] Kiso, M. and Hasegawa, A. (1984) in: Bacterial Endotoxin (Westphal, O. et al. eds) Verlag Chemie-Physik Verlag, Weinheim, in press.
[12] Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N. and Williamson, B. (1975) Proc. Natl. Acad. Sci. USA 72, 3666–3670.
[13] Green, S., Dobrjansky, A., Chiasson, M.A., Carswell, E., Schwartz, M.K. and Old, L.J. (1977) J. Natl. Cancer Inst. 59, 1519–1522.