FEMS Microbiology Letters 20 (1983) 343–346
Published by Elsevier

Structural requirements for initiation of *Limulus* amebocyte lysate gelation by lipoteichoic acids

(Lipoteichoic acid; *Limulus* amebocyte lysate gelation)

Robert E. Kessler *

The Dental Research Institute, School of Dentistry and Department of Microbiology and Immunology, School of Medicine, The University of Michigan, Ann Arbor, MI 48109 USA

Received 20 July 1983
Accepted 21 July 1983

1. SUMMARY

Lipoteichoic acids (LTAs) of varying chemical composition from five streptococcal species and one lactobacillus species initiated gelation of *Limulus* amebocyte lysate. Preincubation with antisera specific for the poly(glycerol phosphate) (PGP) chain or, when appropriate, with antisera, specific for a carbohydrate substituent inhibited gelation initiated by lipoteichoic acid, but did not inhibit lipopolysaccharide-initiated gelation. Higher specific activities were found for those LTAs with higher D-alanine or carbohydrate substitution or with a shortened PGP chain, which suggested that those structural features which decreased the relative hydrophilicity or charge to mass ratio appeared to increase the specific activity.

2. INTRODUCTION

The *Limulus* amebocyte lysate (LAL) assay is widely used as a substitute for the rabbit pyrogen test for detecting contamination of pharmaceuticals and other therapeutic products with pyrogenic endotoxins; i.e., lipopolysaccharides (LPSs) from gram-negative bacteria [1,2]. In clinical medicine, LAL assay results have been shown to correlate well with the presence of gram-negative bacteria in urine and cerebrospinal fluids and cervical exudates (summarized in [3]). Positive LAL reactions with blood samples have been correlated with gram-negative infections, although there are problems with inhibitors in blood [2]. There also is a report of some patients with gram-positive infections yielding positive LAL assay results [4], a finding not observed in other studies [1,3].

Although the reaction of LAL with LPS has been considered to be highly specific, apparently by virtue of the high sensitivity for LPS, other compounds have also been shown to initiate gelation of LAL. Positive reactions have been reported for proteins and polynucleotides [5], peptidoglycans [6], a water-soluble carboxymethylated dextran [7] and lipoteichoic acid (LTA) [8]. The mechanism of gelation is different with the dextran [7]; the mechanism of activation has not been determined for any of the others. In most cases the relative amounts required for gelation are 3–6 orders of magnitude greater than that of highly reactive LPS standards. However, not all LPSs are as active as these standards and a wide range of relative activity may be found [9].

* Present address: The Bristol-Myers Company, Pharmaceutical Research and Development Division, P.O. Box 4755, Syracuse, NY 13221, U.S.A.

LTAs share the common feature of a poly(glycerol phosphate) (PGP) chain, usually 20–30 units in length, that is covalently attached to a lipid moiety. The lipid moiety is usually identical to a membrane glycolipid or phosphatidyl glycolipid [10]. LTAs differ in the extent and type of substitution on the PGP chain. Substitution at the second carbon of the glycerols may be in the form of D-alanine in ester linkage or sugars attached by glycosidic bonds, or both [10]. Highly sugar-substituted LTAs may bear the alanine on a hydroxyl group of the sugar residue [10]. Presented in this report is further documentation for the gelation of LAL in the presence of LTA, and results that demonstrated that differences in LTA structure influenced the minimum amount of LTA required to initiate LAL gelation. It appeared that those structural features which decrease the relative hydrophilicity, or charge to mass ratio of LTA increase the specific activity.

3. MATERIALS AND METHODS

Limulus amebocyte lysate (Limusate, lot nos. 61B1 and 81B1) and LPS from *Escherichia coli* O55 : B5 were obtained from Calbiochem (San Diego, CA). These lots had listed potencies of 0.022 and 0.018 ng/ml, respectively, with FDA reference standard EC2 and tested potencies of 0.2 and 0.1 ng/ml with LPS O55 : B5. The manufacturer's directions for reconstitution, storage and usage were rigorously followed. Sterile pyrogen-free water was used for reconstitution of lyophilized LAL, LPS, LTAs and for all dilutions thereof. Reconstituted LAL was kept on ice and used within 4 h of reconstitution or frozen at −20°C for up to 1 week. All glassware was rendered pyrogen-free by baking at 210°C for 4 h in a dry air oven. The assay was carried out in 10 × 75 mm glass tubes with equal volumes of sample and LAL (0.1 ml). A positive reaction was recorded only for a firm clot which remained in the tube upon inversion after exactly 60 min at 37°C. Endpoints of clotting were determined by assaying 10-fold dilutions and then assaying concentrations between the lowest positive dilution and the highest negative dilution. Every group of tests included a negative control (pyrogen-free water) and a positive control of LPS at the minimum concentration required for gelation. All endpoints reported here were determined with the 81B1 lot; duplicate experiments with the 61B1 lot gave similar results but at approximately 2-fold higher levels.

The LTAs used in these experiments were available from a previous study [11] except for the LTA from *Lactobacillus casei* NCTC 6991 which was kindly provided as a reference standard by Dr. A.J. Wicken, University of New South Wales. Precautions were taken to prevent LPS contamination during growth of the bacteria and during preparation of each LTA; i.e., monitoring cultures for contamination with gram-negative bacteria, baking glassware, and testing of glass distilled water, buffers and column effluents for LAL activity (all negative).

Fatty acid esters were hydrolyzed in 0.2 N KOH in methanol, 15 min at 37°C [13]. The PGP chain of dealanylated group A streptococcal LTA was enzymatically shortened using a phosphodiesterase–phosphomonoesterase mixture prepared as described by Fischer et al. [14]. Progress of the reaction was measured by the release of glycerol (Boehringer-Mannheim, Indianapolis, IN). LTA was separated from free glycerol and enzyme by chromatography on AcA22 [15]. The final chain length was determined by the ratio of phosphorus to glucose [16].

4. RESULTS AND DISCUSSION

The sources and phosphorus : glucose : alanine ratios of the LTAs used in this study are given in Table 1. Glucose content provides a rough estimate of the degree of carbohydrate substitution on these LTAs [11]. All of the glucose of LTAs I and II from the group A streptococci is solely within the glycolipid moiety, but the additional glucose content of LTAs III–VII is found in glycosidic linkage along the PGP chain in the form of kojibiose, kojitriose, or possibly both [18,19].

LTAs with carbohydrate and alanine at approximately the same percentage substitution were nearly equivalent in activity (Table 1, cf. LTAs I and II with III). The presence of alanine on the

Table 1

Lipoteichoic acid compositions and gelation endpoints

LTA [a]	Source	P : Glc : Ala [b]	Gelation endpoint [c] (μg)	Phosphorus equivalents (nmol)
I	Group A Streptococcus (S43)	1 : 0.09 : 0.43	0.5	1.9
II	Group A Streptococcus (2GL318)	1 : 0.07 : 0.29	0.5	1.9
III	Group D streptococcus (*S. faecalis* JH2-2)	1 : 0.38 : 0.34	0.5	1.5
IV	Group D Streptococcus (*S. faecium* ATCC9790)	1 : 1.04 : 0.33	0.25	0.525
V	Group D Streptococcus (*S. faecium* ATCC9790)	1 : 1.05 : 0.03	0.25	0.50
VI	Group D Streptococcus (*S. faecium* ATCC9790)	1 : 1.46 : 0.03	0.25	0.45
VII	Group D Streptococcus (*S. faecium* NCIB8191)	1 : 2.47 : 0.13	0.025	0.012
VIII	*Lactobacillus fermentum* NCTC6991	ND [d]	0.5	ND

[a] Two preparations each of LTAs I, III, IV, V, VII were tested. Results were equivalent for each pair.
[b] Phosphorus : glucose : alanine ratios.
[c] LTA/0.1 ml prior to addition of 0.1 ml LAL.
[d] Not determined.

carbohydrate substituent did not appear to influence the relative activity (cf. LTAs IV and V). The highest activity (LTA VII) appeared to be associated with a high degree of substitution with the longest glycoside chain (a trisaccharide). The equivalent nmol phosphorus for each LTA gelation endpoint is given in Table 1 for purposes of comparison since the ratio of mol LTA/unit dry weight varies with chain length and substitution. Higher concentrations of LTA were required for LAL gelation after removal of alanine from LTAs I and II (Table 2). The LTAs and dealanylated LTAs were compared here on the basis of phosphorus content since chain lengths of native and derivative polymers in this case are the same, and thus equimolar amounts of phosphorus reflect equimolar amounts of the LTAs.

To provide further evidence for LTA-initiation of gelation, and the absence of LPS in LTA preparations, inhibition of gelation by pretreating a moderately substituted LTA and the highly substituted LTA VII with antisera was attempted. At concentrations of LTA close to the minimum

Table 2

Effect of removal of esterified D-alanine from LTA on gelation of *Limulus* amebocyte lysate

LTA	Ala : P	LTA phosphorus in reaction mixture (nmol)			
		19	9.5	3.8	1.9
I	0.43	+ [b]	+	+	+
IA [a]	0.02	+	+	–	–
II	0.29	+	+	+	+
IIA [a]	0.01	+	+	–	–

[a] Derived from LTA with corresponding numeral by dialysis against 0.1 M Tris–HCl buffer (pH 8.0) for 24 h at 37°C and then against distilled water (3 changes) for 24 h at 4°C [12].
[b] +, gelation.

Table 3

Inhibition of *Limulus* amebocyte lysate gelation with antilipoteichoic acid antisera

LTA		Antiserum added [a]		Gelation
No.	Amount (μg/0.1 ml)	Anti-PGP [b] (μl)	Anti-GrpD [c] (μl)	
III	1.0	0	0	+
		2	0	–
	10.0	10	0	+
		10	10	–
VII	0.1	0	0	+
		5	0	–
		0	5	–
	1.0	5	0	+
		0	5	+
		10	10	–
None		10	0	–
		0	10	–

[a] Antisera were added to LTA and the mixture incubated for 15 min at 37°C prior to addition of LAL (0.1 ml).
[b] Antiserum prepared against LTA from *L. casei* NCTC6375 and demonstrated to be PGP-specific by Dr. K.W. Knox.
[c] Streptococcal group D antiserum (specific for group D LTA since LTA is the group D antigen [17]).

amount required for gelation, inhibition of gelation was possible with antisera directed against either the PGP backbone alone or antiserum directed predominantly against the substituents of the group D LTA (Table 3). At higher concentrations of LTA, a combination of both antisera was effective. Neither antisera inhibited LPS-initiated LAL gelation at concentrations of 0.1–10 ng/ml LPS.

Removal of fatty acids by mild base hydrolysis increased the minimum amount of LTA III required for LAL gelation from 0.5 μg to > 20 μg. No gelation was observed at 20 μg, but a positive reaction was seen at 200 μg, which was the next highest concentration tested. Since the base-treated LTA was also rechromatographed, it is not likely that there was any residual, partially deacylated LTA remaining. Thus it would appear that intact fatty acids are not required for LAL activation, although they certainly augment the activity. This may be related to differences in physical structure; i.e., formation of vesicular aggregates of LTA but not deacylated LTA.

LTA II was dealanylated (LTA IIA), then shortened enzymatically, and rechromatographed yielding a preparation with a reduced average chain length of 14.5 (LTA IIB) as compared to the original average chain length of 28. The minimum amount required for LAL gelation decreased from 9.5 nmol LTA phosphorus (2.15 μg) of LTA IIA (Table 2) to 0.49 nmol LTA phosphorus (0.25 μg) of the shortened LTA IIB. Since there are approximately 2.1-times as many molecules of LTA IIB per unit mass as there are of LTA IIA, reduction in chain length resulted in a net 4-fold increase in specific activity on a molar basis. Although the effect of shortening the PGP chain further and the minimum chain lengths for LAL activation were not determined, neutralized acid hydrolysates were negative at 50 μg of material.

In summary, specific antibody inhibition of LTA-initiated gelation but not LPS-initiated gelation provided definitive evidence for LTA-initiated gelation of LAL. The apparently higher specific activities found with higher substituted LTAs or LTA with decreased chain length suggested that those structural features which decreased the relative hydrophilicity or charge to mass ratio appeared to increase the specific activity of the LTA molecule for initiation of the LAL clotting mechanism.

ACKNOWLEDGEMENTS

This research was supported by United States Public Health Service Grant DE02731. I thank Dr. K.W. Knox, Institute of Dental Research, Sydney, Australia and Dr. L. Pine, Center for Disease Control, Atlanta, Georgia, USA for generously providing antisera with specificity for PGP and the streptococcal group D antigen, respectively.

REFERENCES

[1] Cooper, J.F. (1979) in Biomedical Applications of the Horseshoe Crab (Limulidae) (Cohen, E., Ed.) pp. 345–352, Liss, New York.
[2] Levin, J. (1979) in Biomedical Applications of the Horseshoe Crab (Limulidae) (Cohen, E., Ed.) pp. 235–244. Liss, New York.
[3] Fink, P.C., Lehr, L., Urbaschek, R.M. and Kozak, J. (1981) Klin. Wochenschr. 59, 213–218.
[4] Stumacher, R.J., Kovnat, M.J. and McCabe, W.R. (1973) N. Engl. J. Med. 288, 1261–1269.
[5] Elin, R.J. and Wolff, S.M. (1973) J. Infect. Dis. 128, 349–352.
[6] Wildfeuer, A., Heymer, B., Spilker, D., Schleifer, K.-H., Vanek, E. and Haferkamp, O. (1975) Z. Immun. Forsch. Bd. 149, 258–264.
[7] Kakinuma, A., Asano, T., Torii, H. and Sugino, Y. (1981) Biochem. Biophys. Res. Comun. 101, 434–439.
[8] Fine, D.H., Kessler, R.E., Tabak, L.A. and Shockman, G.D. (1977) J. Dent. Res. 56, 1500.
[9] Elin, R.J., Sandberg, A.L. and Rosenstreich, D.L. (1976) J. Immunol. 117, 1238–1242.
[10] Wicken, A.J. and Knox, K.W. (1980) Biochim. Biophys. Acta 604, 1–26.
[11] Kessler, R.E. and Thivierge, B.H. (1983) Infect. Immun. 41, 549–555.
[12] Fischer, W., Koch, H.U., Rosel, P. and Fiedler, F. (1980) J. Biol. Chem. 255, 4557–4562.
[13] Wicken, A.J., Gibbens, J.W. and Knox, K.W. (1973) J. Bacteriol. 113, 365–372.
[14] Fischer, W., Koch, H.U., Rosel, P., Fiedler, F. and Schmuck, L. (1980) J. Biol. Chem. 255, 4550–4556.
[15] Jacques, N.A., Hardy, L., Campbell, L.K., Knox, K.W., Evan, J.D. and Wicken, A.J. (1979) Infect. Immun. 26, 1079–1087.
[16] Kessler, R.E., van de Rijn, I. and McCarty, M. (1979) J. Exp. Med. 150, 1498–1509.
[17] Wicken, A.J., Elliott, S.D. and Baddiley, J. (1963) J. Gen. Microbiol. 31, 231–239.
[18] Wicken, A.J. and Baddiley, J. (1963) Biochem. J. 87, 54–62.
[19] Fischer, W., Rosel, P. and Koch, H.U. (1981) J. Bacteriol. 146, 467–475.