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Investigations on the Specificity of the Limulus Test for the Detection of Endotoxin

ALEXANDER WILDFEUER, BERNO HEYMER, KARL H. SCHLEIFER, AND OTTO HAFERKAMP

Department of Pathology I, University of Ulm, Ulm, and Botanic Institute, University of Munich, Munich, West Germany

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Lysates obtained from amoebocytes of *Limulus polyphemus*, the horseshoe crab, showed gel formation after the addition of bacterial endotoxin. In contrast to living gram-negative bacteria, viable gram-positive microorganisms did not cause gelation of lysate. Nevertheless, peptidoglycan isolated from the cell walls of various gram-positive organisms did induce the reaction. However, the activity of peptidoglycan was 1,000 to 400,000 times less than that of *Escherichia coli* lipopolysaccharide. After exposure to lysozyme, peptidoglycan no longer gelled amoebocyte lysate, therefore apparently excluding endotoxin contamination. Gelation of amoebocyte lysate by endotoxin or peptidoglycan was inhibited by different concentrations of sodium polystyrolsulfonate. Whereas these studies confirm the specificity of the Limulus test for bacterial endotoxins, they also indicate that other substances of bacterial origin should be investigated for their ability to gel amoebocyte lysate.

The Limulus test, described by Levin and Bang (10, 11), is currently the simplest and most sensitive method for the detection of gram-negative bacterial endotoxins (15, 22). The assay is based on the ability of endotoxins to induce gelation of a lysate obtained from the amoebocytes of *Limulus polyphemus*, the horseshoe crab. Lysate gelation is comparable to blood clotting in mammals (10, 11). The Limulus assay permits the detection of endotoxin in concentrations as little as 5×10^{-4} μg/ml (13). Because of this great sensitivity, recent attempts have been made to use the test for diagnostic purposes, e.g., for the determination of endotoxemia in patients with sepsis and/or hypotension (2, 12). However, whether lysate gelation is induced only by endotoxin (i.e., is specific for the lipopolysaccharides of gram-negative bacteria) or whether it also may occur in the presence of other bacterial substances has not been reported previously. It would seem pertinent to evaluate this possibility, since it has been found that gram-positive bacteria do possess components, the so-called cell wall peptidoglycans, which have endotoxin-like properties (7, 16). In addition, disseminated intravascular coagulation, shock, and the Waterhouse-Friderichsen syndrome have been observed occasionally in sepsis due to gram-positive bacteria (1, 5). Therefore, in the present studies various gram-positive bacterial peptidoglycans were tested for activity in the Limulus assay.

MATERIALS AND METHODS

Bacteria. The following bacterial strains were used in these studies: group A *Streptococcus* strains B196 (type 17), T22 (type 22), A374 (type 12), and B514 (type 50); *Staphylococcus aureus* strain Copenhagen; *Staphylococcus epidermidis* strain 24; *Micrococcus lysodeikticus* strain ATCC 4698; *Escherichia coli* O111; *Salmonella typhimurium*; *Serratia marcescens*; *Klebsiella pneumoniae* strain ATCC 10031; *Enterobacter aerogenes*; and *Pasteurella multocida*.

Endotoxin. The endotoxin preparation (lipopolysaccharide W, *E. coli*, O127:B8) was obtained from Difco Laboratories, Detroit, Mich.

Peptidoglycan. To avoid possible contamination with endotoxin, peptidoglycan was routinely prepared using pyrogen-free glassware and solutions (23). Group A streptococcal peptidoglycan was obtained by four successive formamide extractions of trypsinized cell walls, as described previously (6). The particulate matter was solubilized by sonication (6). The chemical composition and immunological characteristics of such preparations have previously been reported (8). Peptidoglycans of *S. aureus*, *S. epidermidis*, and *M. lysodeikticus* were isolated from their respective trypsinized cell walls by two successive 3-h extractions with trichloroacetic acid (10%) at 60 C and subsequently solubilized by sonication (6). The chemical and immunological properties of such preparations also have been reported in detail (17-20).

Amoebocyte lysate. *L. polyphemus* was obtained from the Marine Biological Laboratory, Woods Hole,

Mass. Isolation of amoebocytes and lysate production were performed as described by Levin et al. (11–13). In addition, some lysate purchased from the Marine Biological Laboratory, in lyophilized form, was used. In each instance, activity of the various lysate batches was determined with the same endotoxin preparation (lipopolysaccharide W, *E. coli*, O127:B8).

Limulus test. Various concentrations (see tables) of washed living bacteria, endotoxin, or peptidoglycan in 0.1 ml of pyrogen-free saline were mixed with the same volume of amoebocyte lysate in sterile, pyrogen-free, rubber-stoppered glass tubes (10 by 80 mm), incubated at 37 C for 1 h, refrigerated for 15 min at 4 C, and then read. Results were recorded as solid gel, incomplete gel, definite increase in opacity and viscosity, or no reaction.

Controls. The following controls were incorporated routinely in each test: one tube contained 0.1 ml of amoebocyte lysate only, one tube contained 0.1 ml of lysate and 0.1 ml of pyrogen-free saline, and one tube contained 0.1 ml of lysate and 0.0005 μg of *E. coli* lipopolysaccharide in 0.1 ml of pyrogen-free saline.

Since traces of endotoxin can produce lysate gelation, it was important to prove that activity of peptidoglycan in the Limulus assay was not due to endotoxin contamination. Whereas lysozyme hydrolyzes peptidoglycan polymers (6, 8, 16), it does not attack endotoxin (9). On the other hand, treatment of endotoxin with alkali strongly reduces the activity in the Limulus assay (13, 22). Therefore, the following experiments were performed: (i) Samples (1 ml) of all peptidoglycan preparations (4 mg/ml), as well as of endotoxin (200 μg/ml), were treated with 3× crystallized egg white lysozyme (Sigma, St. Louis, Mo.) at a concentration of 100 μg/mg. Peptidoglycan or endotoxin were dissolved in 0.15 ml of saline, pH 6.2, incubated with lysozyme at 37 C for 18 h, and then checked for activity in the Limulus test. (ii) Samples (1 ml) of endotoxin (200 μg/ml) and peptidoglycan (4 mg/ml) were treated with 0.25 ml of NaOH at 56 C for 60 min (14), neutralized, and subsequently tested for ability to produce lysate gelation. To determine that lysozyme and NaOH treatment of peptidoglycan was effective, systems independent from activities measured in the Limulus assay were employed. This was achieved by use of the capillary precipitin test (21) with rabbit anti-peptidoglycan antisera (6, 8).

Finally, experiments were performed to determine whether inhibitors of the endotoxin-lysate reaction also blocked the peptidoglycan-induced lysate gelation. For this purpose, the polyanion, sodium polystyrosulfonate (Serva, Heidelberg, West Germany), in concentrations from 0.00005 to 50.0 μg per assay, was added to tubes containing lysate and peptidoglycan or endotoxin.

RESULTS

Amoebocyte lysate prepared in our own laboratories, as well as lysate obtained from a commercial source, permitted detection of endotoxin (*E. coli* lipopolysaccharide) at 0.0005 μg/0.1 ml of test solution.

TABLE 1. *The effect of living bacteria in the Limulus test (10⁸ organisms per test)*

Bacteria	Limulus test[a]
Gram positive	
Streptococcus group A (4 strains)	–
Staphylococcus aureus	–
Staphylococcus epidermidis	–
M. lysodeikticus	–
Gram-negative	
E. coli	+++
Serratia marcescens	+++
Salmonella typhimurium	+++
K. pneumoniae	+++
E. aerogenes	+++
P. multocida	+++

[a] +++, Solid gelation; ++, incomplete gelation; +, increased opacity and viscosity; –, no reaction.

Screening of a variety of bacterial species in the Limulus test showed that only gram-negative bacteria induced gelation of amoebocyte lysate (Table 1). These results were obtained by using washed, living organisms at a concentration of 10^8 per assay. The major observations of the present studies are summarized in Table 2, in which gelling activity of *E. coli* lipopolysaccharide and peptidoglycans from several bacterial species are compared. Effects of lysozyme and NaOH treatment on these substances are presented simultaneously. It should be emphasized that the Limulus assay and the capillary precipitin test measure different porperties of peptidoglycans. The data (Table 2) indicate that both *E. coli* lipopolysaccharide and various peptidoglycans initiated gelation of amoebocyte lysate. However, concentrations of peptidoglycans required to produce a positive Limulus test were 1,000 to 400,000 times higher than for endotoxin. As expected (9), treatment with lysozyme did not reduce the ability of endotoxin to gel amoebocyte lysate, whereas exposure to NaOH resulted in a profound loss of activity. In contrast, lysozyme treatment of peptidoglycans caused complete inactivation of gelling potency. Weak alkaline hydrolysis also resulted in inactivation of peptidoglycan. Effects of lysozyme and NaOH treatment of peptidoglycans were readily demonstrable by capillary precipitation (Table 2). Exposure to lysozyme destroyed precipitating activity of all peptidoglycan preparations tested. In contrast, NaOH treatment produced this effect in staphylococcal peptidoglycan only.

Results of inhibition studies are presented in Table 3. Sodium polystyrolsulfonate was a po-

TABLE 2. *Activity of endotoxin or peptidoglycan in the Limulus and capillary precipitin tests*

Material (μg[a])	Limulus test[b]			Capillary precipitin test[c]		
	No pretreatment	Lysozyme treated	NaOH treated	No pretreatment	Lysozyme treated	NaOH treated
Lipopolysaccharide *E. coli*						
1.0	+++	+++	+++	ND	ND	ND
0.1	+++	+++	−			
0.01	+++	+++				
0.0005	+++	+++				
0.00005	−	−				
Peptidoglycan *Streptococcus* group A						
400.0	+++	−	−	++	−	++
200.0	+++					
100.0	+					
40.0	−					
Peptidoglycan *Staphylococcus aureus*						
150.0	+++	−	−			
75.0	+++			++	−	−
37.5	+					
15.0	−					
Peptidoglycan *Staphylococcus epidermidis*						
200.0	+++	−	−	+++	−	−
100.0	−					
Peptidoglycan *M. lysodeikticus*						
30.0	+++	−	−	++	−	++
3.0	+++					
0.3	+++					
0.03	−					

[a] Concentration per test.
[b] +++, Solid gelation; ++, incomplete gelation; +, increased opacity and viscosity; −, no reaction.
[c] +++, Very strong reaction; ++, strong reaction; +, weak reaction; −, no reaction.
[d] ND, Not done.

tent inhibitor of endotoxin, as well as peptidoglycan-induced lysate gelation. However, the small amount (10^{-5} μg) sufficient to inhibit lysate gelation by 200 μg of peptidoglycan demonstrates the weak activity of peptidoglycan in the Limulus assay. In contrast, 0.5 μg was required to block the activity of only 2.5 μg of endotoxin.

Formamide extraction of *E. coli* endotoxin, under conditions (30 min at 180 C) used to prepare streptococcal peptidoglycan, hydrolyzed 97% of the endotoxin. The remaining 3% exhibited gelation activity only 1/10,000 that of the native parent endotoxin.

DISCUSSION

The present studies indicate that endotoxins of gram-negative bacteria and peptidoglycans of gram-positive organisms both induce gelation of lysates prepared from the amoebocytes of *L. polyphemus*. However, the activity of peptidoglycans is 1,000 to 400,000 times less than that of endotoxin. Furthermore, in contrast to living gram-negative bacteria, endotoxin, and isolated peptidoglycans, intact gram-positive bacteria did not induce lysate gelation. Therefore, the present studies substantiate the specificity of the Limulus test for the detection of bacterial endotoxins. These findings are in agreement with the clinical observations that the Limulus test is negative in patients with sepsis due to gram-positive organisms (2, 3, 12). The high concentrations of peptidoglycan that were required to gel amoebocyte lysate are most unlikely to be present in clinical situations.

Treatment with lysozyme completely de-

TABLE 3. *Inhibition of endotoxin or peptidoglycans in the Limulus test by sodium polystyrolsulfonate*

Material (μg^a)	Sodium polystyrol-sulfonate (μg^a)	Limulus test[b]
Lipopolysaccharide *E. coli*		
0.0005	0	+++
2.5	5.0	−
2.5	0.5	+
2.5	0.05	++
2.5	0.00005	++
Peptidoglycan *Streptococcus pyogenes* A		
200		+++
100		+++
50		+
20		−
200	0.00005	−

[a] Concentration per test.

[b] +++, Solid gelation; ++, incomplete gelation; +, increased opacity and viscosity; −, no reaction.

stroyed the gelation activity of peptidoglycan. This loss of activity after exposure to an enzyme which hydrolyzes mucopeptide polymers, but does not attack endotoxin, apparently excludes endotoxin contamination as a possible explanation for peptidoglycan gelling potency. Exposure of endotoxin or peptidoglycans to NaOH destroyed gelation activity of both substances and, therefore, did not permit further evaluation of the possibility that contamination of peptidoglycans by small concentrations of endotoxin accounted for the reaction between preparations of peptidoglycan and amoebocyte lysate.

Elin and Wolff reported that synthetic polynucleotides reacted with amoebocyte lysate (4). They apparently ruled out contamination of the polynucleotides by bacterial endotoxins by the demonstration that incubation of polynucleotides with ribonuclease eliminated the ability of the polynucleotide to gel amoebocyte lysate, whereas ribonuclease did not alter the reactivity of endotoxin with amoebocyte lysate. Our studies suggest that not only endotoxin but other bacterial components should be investigated for their ability to initiate the gelation of amoebocyte lysate (23).

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LITERATURE CITED

1. Bisno, A. L., and J. C. Freeman. 1970. The syndrome of asplenia, pneumococcal sepsis, and disseminated intravascular coagulation. Ann. Int. Med. **72**:389–393.
2. Caridis, D. T., R. B. Reinhold, P. W. H. Woodruff, and J. Fine. 1972. Endotoxaemia in man. Lancet **1**:1381–1385.
3. Das, J., A. A. Schwartz, and J. Folkman. 1973. Clearance of endotoxin by platelets: role in increasing the accuracy of the Limulus gelation test and in combating experimental endotoxemia. Surgery **74**:235–240.
4. Elin, R. J., and S. M. Wolff. 1973. Nonspecificity of the Limulus amebocyte lysate test: positive reactions with polynucleotides and proteins. J. Infect. Dis. **128**:349–352.
5. Grant, M. D., H. I. Horowitz, V. Lorian, and H. R. Brodman. 1970. Waterhouse-Friderichsen syndrome induced by pneumococcemic shock. J. Amer. Med. Ass. **212**:1373–1374.
6. Heymer, B. 1972. Untersuchungen über die enzymatische Freisetzung bakterieller Antigene. *In* H. Schaefer (ed.), Theoretische und klinische Medizin in Einzeldarstellungen, vol. 56. Huthig-Verlag, Heidelberg.
7. Heymer, B., B. Bültmann, and O. Haferkamp. 1971. Toxicity of streptococcal mucopeptides *in vivo* and *in vitro*. J. Immunol. **106**:858–861.
8. Heymer, B., W. Schachenmayer, B. Bültmann, R. Spanel, O. Haferkamp, and W. C. Schmidt. 1973. A latex agglutination test for measuring antibodies to streptococcal mucopeptides. J. Immunol. **111**:478–484.
9. Lehrer, S., and A. Nowotny. 1972. Isolation and purification of endotoxin by hydrolytic enzymes. Infect. Immunity **6**:928–933.
10. Levin, J., and F. B. Bang. 1964. The role of endotoxin in the extracellular coagulation of Limulus blood. Bull. Johns Hopkins Hosp. **115**:265–274.
11. Levin, J., and F. B. Bang. 1968. Clottable protein in Limulus: its localization and kinetics of its coagulation by endotoxin. Thromb. Diath. Haemorrh. **19**:186–197.
12. Levin, J., T. E. Poore, N. P. Zauber, and R. S. Oser. 1970. Detection of endotoxin in the blood of patients with sepsis due to gram-negative bacteria. N. Engl. J. Med. **283**:1313–1316.
13. Levin, J., P. A. Tomasulo, and R. S. Oser. 1970. Detection of endotoxin in human blood and demonstration of an inhibitor. J. Lab. Clin. Med. **75**:903–911.
14. Neter, E., O. Westphal, O. Lüderitz, E. A. Gorzynski, and E. Eichenberger. 1956. Studies of enterobacterial lipopolysaccharides. J. Immunol. **76**:377–385.
15. Rojas-Corona, R. R., R. Skarnes, S. Tamakuma, and J. Fine. 1969. The Limulus coagulation test for endotoxin. A comparison with other assay methods. Proc. Soc. Exp. Biol. Med. **132**:599–601.
16. Rotta, J. 1969. Biological activity of cellular components of group A streptococci *in vivo*. Curr. Top. Microbiol. Immunol. **48**:63–101.
17. Schleifer, K. H., L. Huss, and O. Kandler. 1969. Die Beeinflussung der Aminosäuresequenz des serinhaltigen Mureins von Staphylococcus epidermidis Stamm 24 durch die Nährbodenzusammensetzung. Arch. Mikrobiol. **68**:387–404.
18. Schleifer, K. H., and O. Kandler. 1967. Micrococcus lysodeikticus: a new type of cross-linkage of the murein. Biochem. Biophys. Res. Commun. **28**:965–972.
19. Schleifer, K. H., and R. M. Krause. 1971. The immunochemistry of peptidoglycan. Separation and characterization of antibodies to the glycan and to the peptide subunit. Eur. J. Biochem. **19**:471–478.
20. Schleifer, K. H., and R. M. Krause. 1971. The immunochemistry of peptidoglycan. I. The immunodominant

site of the peptide subunit and the contribution of each of the amino acids to the binding properties of the peptides. J. Biol. Chem. **246**:986–993.
21. Swift, H. F., A. T. Wilson, and R. C. Lancefield. 1943. Typing group A hemolytic streptococci by M precipitin reactions in capillary pipettes. J. Exp. Med. **78**:127–133.
22. Yin, E. T., C. Galanos, S. Kinsky, R. A. Bradshaw, S. Wessler, O. Lüderitz, and M. E. Sarmiento. 1972. Picogram-sensitive assay for endotoxin: gelation of Limulus polyphemus blood cell lysate induced by purified lipopolysaccharides and lipid A from gram-negative bacteria. Biochim. Biophys. Acta **261**:284–289.
23. Young, N. S., J. Levin, and R. A. Prendergast. 1972. An invertebrate coagulation system activated by endotoxin: evidence for enzymatic mediation. J. Clin. Invest. **51**:1790–1797.