Factors Affecting the Sensitivity of *Limulus* Lysate

JAMES D. SULLIVAN, JR., AND STANLEY W. WATSON

Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

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*Limulus* lysate clots when mixed with picogram quantities of endotoxins. The sensitivity of the lysate was improved 100-fold by the removal of an inhibitor and addition of divalent cations. The methods developed in this investigation eliminated much of the seasonal variability of the lysate, improved the heat stability after lyophilization, and made it possible to use the lysate with saline solutions.

*Limulus* lysate (an aqueous extract of the amebocytes obtained from the blood of the horseshoe crab, *Limulus polyphemus*) clots when mixed with picogram quantities of endotoxins which are lipopolysaccharide components of the outer cell-wall layer of gram-negative bacteria. Since the initial description of the lysate method for the detection of endotoxins (7), it has become apparent that this biologically active material may serve as a useful tool in several scientific disciplines. It has already been used for the detection of endotoxemia (6, 8) and bacteriuria (4), and for the diagnosis of gram-negative spinal meningitis (10). The use of the *Limulus* test for the detection of endotoxins in radiopharmaceuticals and biologicals has already been demonstrated (2) and in the future it may be routinely used by pharmaceutical companies for this purpose. It is foreseeable that this test can be used to determine pollution in natural waters and to detect gram-negative bacterial contamination in food products. Lysate has been used in our laboratory to measure the horizontal and vertical distribution of bacteria in oceanic waters. As a laboratory tool, *Limulus* lysate may be useful in studying why endotoxins are so biologically active and for obtaining a better understanding of the biochemical events which occur in primitive clotting systems.

Before the full potential of *Limulus* lysate can be realized, the biochemical mechanism involved in the lysate-endotoxin reaction must be more fully understood and the factors affecting this reaction must be detailed. Young et al. (16) have shown the reaction to be enzmyatic consisting of endotoxin activation of a high-molecular-weight enzyme followed by its conversion of a low-molecular-weight clottable protein to a gel. Solum (14, 15) has purified and characterized the clottable protein and gel protein.

One of the serious objections to the use of the *Limulus* lysate method for the detection of endotoxins stemmed from the fact that the biological activity of the lysate varied from batch to batch and the factors controlling this variability were not understood. This report provides an explanation for the variable potency of lysate and corrective methods for improvement.

**MATERIALS AND METHODS**

**Preparation of Limulus lysate.** Horseshoe crabs were obtained from the Woods Hole Marine Biological Laboratory. Crabs were bled three to five times per week with an average of 50 crabs per bleed. Lysate was prepared by the method of Jorgensen and Smith (5).

**Assay of lysate.** To test the sensitivity of the lysate, 0.1 ml of lysate was mixed with 0.1 ml of endotoxin in a pyrogen-free disposable test tube (10 by 75 mm) (Becton-Dickinson, Rutherford, N.J.). This mixture was incubated at 37°C for 1 h and scored as positive if the lysate formed a firm clot which would not break when the tube was slowly inverted 180 degrees.

**Endotoxins.** A *Klebsiella pneumoniae* standard endotoxin preparation was furnished by the Food and Drug Administration and contained 100 ng of endotoxin per ml when reconstituted with 10 ml of pyrogen-free distilled water (Travenol Laboratories, Deerfield, II.). From this stock solution, which is stored at 5°C, a 1-ng/ml solution was prepared freshly each day with pyrogen-free water and from which a dilution series was made.

Other endotoxin standards although not routinely used were *Escherichia coli* 0127:B8 (Difco) and *Salmonella minnesota* 9700 (Difco).

**Removal of inhibitor from lysate.** Equal volumes of lysate and organic solvents were shaken for 1 h on a rotary shaker at 5°C. After shaking, the mixture was centrifuged (4,000 × g) and the aqueous phase was withdrawn and titered with various concentrations of endotoxin. All solvents used were reagent grade.
Lyophilization and storage of the lysate. Before lyophilizing, 5 ml of lysate was dispensed into 10-ml serum vials equipped with a split rubber stopper. The lysate was then frozen at \(-74 \) C, lyophilized, and sealed under 10-\( \mu \)m vacuum in a Virtis 10-800 freeze-dryer (Gardiner, N.Y.) equipped with a stoppering plate. Usually 99 vials of lysate were dried at a time with a drying time of approximately 36 h. The moisture content of dried lysate was 2%. After drying the vials were banded with metal seals and stored at \(-74 \) C.

Polyacrylamide gel electrophoresis. To demonstrate the nature of the inhibitor, samples of lysate before and after chloroform extraction were subjected to polyacrylamide disc gel electrophoresis at pH 9.5 according to Ornstein (11) and Davis (3). Gels were fixed in 12.5% trichloroacetic acid and stained with a 1:20 dilution in 12.5% trichloroacetic acid of a 1% solution of Coomassie blue R (Sigma Chemical Co., St. Louis, Mo.) according to Chrambach et al. (1). A duplicate gel was stained for lipoprotein by immersing it in a saturated solution of Sudan black B (Matheson, Coleman, and Bell, East Rutherford, N.J.) in ethylene glycol (13). Gels were destained in the first case with 12.5% trichloroacetic acid and in the latter with ethylene glycol.

RESULTS

Using procedures for lysate preparation similar to those described by Jorgensen and Smith (5) and Reinhold and Fine (12) the sensitivity of the lysate prepared in this laboratory varied significantly throughout the year. When these methods were used, 90% of the lysate prepared in our laboratory in the summer of 1972 formed a firm gel with 0.1 to 1.0 ng of endotoxin per ml. Yet in the summer of 1973 and 1974 very little of the lysate prepared would clot with this range of endotoxin. Lysate prepared during the winter of 1973 would not even gel with 100 ng of endotoxin per ml.

The sensitivity of the lysate was improved and much of the variability eliminated by removing an inhibitor. This inhibitor was removed with a variety of water-immiscible organic solvents. Listed in their order of effectiveness they are as follows: chloroform \( > \) ethylene chloride \( > \) methylene chloride \( > \) ethyl ether \( > \) carbon tetrachloride \( > \) trichloroethylene \( > \) toluene \( > \) hexane. Solvents which did not effectively remove the inhibitor include n-butanol, isoamyl alcohol, phenethyl alcohol, amyl acetate, methyl isobutyl ketone, dichlorobenzene, and benzaldehyde. All solvents were shown to be free of endotoxin. Since chloroform was most effective it was routinely used in these experiments.

Even after chloroform extraction the lysate did not achieve maximal sensitivity until a divalent cation was added. Addition of 0.02 M Ca\(^{2+}\), Mg\(^{2+}\) or Mn\(^{2+}\) were equally effective in increasing the sensitivity of the lysate. NaCl (0.154 M) was also added to decrease the turbidity of the blank. The effect of chloroform extraction and addition of divalent cations to the lysate is summarized in Table 1.

Chloroform extraction and addition of divalent cations did not completely eliminate the seasonal variability of the lysate. The lysate prepared during the summer months was more sensitive than that prepared during the winter. In the summers of 1973 and 1974 the lysate clotted with 0.02 to 0.08 ng of endotoxin per ml while the lysate prepared during the winter of 1973-1974 required 0.1 to 0.4 ng of endotoxin per ml for clot formation.

Chloroform extraction altered the stability of the lysate. Before chloroform extraction lysate was stored for periods up to 12 months at \(-74 \) C without loss of activity. Chloroform-extracted lysate occasionally but not always decreased in activity after 3 to 6 months of storage at \(-74 \) C. Nonchloroform-extracted lysate was stable at 5 C for more than a week. Chloroform-extracted lysate was unstable at room temperature and frequently lost activity when stored at 5 C onefield. To avoid loss of activity the chloroform-extracted lysate was routinely kept on ice during the working day. Chloroform-extracted lysate when lyophilized was more stable than nonchloroform-extracted lysate. Lyophilized lysate has been stored in this laboratory for a period of more than 12 months without loss of activity. No loss of activity of lyophilized lysate was found even when stored at 37 C for 2 months. Dried lysate has been shipped without refrigeration from this laboratory to other laboratories in both Europe and United States and investigators receiving the dried lysate reported that it had not deteriorated during shipping.

When chloroform-extracted lysate was used with solutions containing 0.154 M NaCl or

| Table 1. Effect of chloroform extraction and addition of salts on lysate sensitivity |
|----------------------------------|
| Treatment of lysate | Endotoxin (ng/ml) required for clot formation* |
|---------------------|----------------------------------|
| Before chloroform extraction |                                      |
| No added salts                  | 6                                 |
| 0.02 M CaCl\(_2\) and 0.154 M NaCl added | 1                                  |
| After chloroform extraction    |                                      |
| No added salts                  | 1                                 |
| 0.02 M CaCl\(_2\) and 0.154 M NaCl added | 0.04                               |

* Results typical for lysate prepared during summer of 1974.
greater, the sensitivity of the lysate was frequently decreased. When 0.02 M Ca\(^{2+}\) or other divalent cations were added to the chloroform-extracted lysate the sodium inhibition was eliminated.

The fate of the inhibitor during chloroform treatment is not completely understood. When lysate was treated with chloroform, a large precipitate formed particularly at the interface. It appears that the inhibitor was precipitated and/or denatured by solvent treatment. When the residue from evaporation of the chloroform phase was resuspended in 0.154 M NaCl and tested with treated lysate, no inhibition was found.

Comparison of gels stained with Coomassie blue failed to show a difference in protein pattern between chloroform and nonchloroform-treated lysate. A distinction could be seen if gels were stained with Sudan black B. Nontreated lysate showed a single band stainable with Sudan black (mobility = 0.13 relative to bromphenol blue, the tracking dye); the chloroform-treated lysate lacked such a band. Since this compound was water soluble and stained with Sudan black B, it is likely a lipoprotein.

**DISCUSSION**

In our laboratory it was found that high quality lysate could not be routinely prepared from horseshoe crabs collected on Cape Cod on a year-round basis using methods previously described in the literature. The use of the supplemental procedures which we used may not be necessary if the horseshoe crabs are collected during the summer months from other geographic localities. In our laboratory chloroform extraction and the addition of Ca\(^{2+}\) improved the sensitivity of the lysate more than 100-fold and has made it possible to prepare lysate on a year-round basis.

All evidence indicates that chloroform extraction removes an inhibitor but the role of such an inhibitor is not clear. Higher concentrations of inhibitor may be present in the blood of the crab in the winter than in the summer months but this has not been experimentally verified. Possibly the concentration of the inhibitor remains relatively constant while the concentration of the clotting enzyme and/or the clottable protein is decreased during various times of the year. If this is the case then even small amounts of inhibitor might prevent clotting of the lysate during periods when the concentration of active proteins is low.

The in vivo function of the inhibitor in *Limulus* blood is not known. Quite possibly it acts at one or more steps involved in the clotting reaction which have been detailed by Young et al. (16) and include enzyme activation by endotoxin followed by enzymatic conversion of clottable protein to a gel. The inhibitor could limit the activation of clotting enzyme by tying up endotoxin therefore reducing the lysate’s sensitivity. Levin et al. (9) have shown serum of human blood to contain a protein which binds endotoxin reversibly thus interfering with detection of endotoxin either added to or already present in human blood. Endotoxin could be detected with *Limulus* lysate when human serum or plasma was extracted with chloroform (9). The inhibitor in human blood is chloroform sensitive as is the inhibitor in *Limulus* blood and both may function as endotoxin-binding substances. Another possibility is that the inhibitor in *Limulus* blood interferes with the action of endotoxin-activated enzyme on clottable protein. Solum (15) has reported that trypsin in the absence of endotoxin gelled lysate or purified clottable protein. Trypsin inhibitor will block the trypsin-catalyzed reaction but not the endotoxin-dependent reaction (15). The clotting enzyme may be similar to trypsin in action and controlled by an inhibitor highly specific like trypsin inhibitor. Interference with the polymerization of gel protein into a gel may be another possible function of the inhibitor. That more than one inhibitor, each chloroform sensitive, is involved cannot be ruled out.

Preliminary studies on the nature of the inhibitor in *Limulus* blood suggest that it is a lipoprotein. Polycrylamide disc gel electrophoresis of lysate before and after chloroform extraction showed the disappearance of a protein band stainable with Sudan black, a lipoprotein stain, after solvent treatment. Additional experiments are in progress to confirm the lipoprotein nature of the inhibitor.

The addition of divalent cations to lysate increases its sensitivity following solvent treatment. CaCl\(_2\) (0.02 M) or MgCl\(_2\) (0.02 M) appear equally effective in lysate improvement. The role divalent cations play in the lysate-endotoxin reaction is not known. Calcium could function at the enzyme activation step in conjunction with endotoxin, the reaction of activated enzyme with clottable protein, or the polymerization step resulting in a gel. Further studies are needed to determine the site(s) of action for divalent cations in the clotting reaction.
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