Preparation, Sensitivity, and Specificity of Limulus Lysate for Endotoxin Assay

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Limulus amoebocyte lysate was prepared from a total of 180 crabs during 1971 and 1972 by using a slightly modified lysate preparation procedure. Marked variability of lysate potency was noted both years. In addition, lysate quality appeared diminished in 1972 as compared with 1971. Different lysate batches were evaluated for potency by using a variety of endotoxin preparations. Variations in batch potencies were observed, but little variation in reactivity among different endotoxin preparations was noted. Use of potent lysate batches allowed detection of endotoxin concentrations as low as 100 pg/ml. No endotoxin-like activity was observed from 11 different strains of yeast by use of the Limulus assay.

Levin and Bang originally described the ability of amoebocyte lysate from Limulus polyphemus, the horseshoe crab, to form a gel in the presence of minute amounts of endotoxin (6). Since that time, the Limulus assay has been used as a method of detecting endotoxin in patients with gram-negative septicemia (1, 7, 8), for the study of experimental endotoxemia and shock in animals (4) and as a method of detecting pyrogen in parenteral pharmaceuticals (3). This method has been shown to be the most sensitive method presently available for the detection of endotoxin (2, 9). However, workers differ as to the source of the crabs used for lysate production, the methods of preparing lysate, and the endotoxin standard used to determine lysate sensitivity. Furthermore, Lindberg et al. (R. B. Lindberg, et al., Fed. Proc., p. 791, 1972) reported significant variations in activity of lysate pools which apparently were due to seasonal influences on the crabs. Recently, methods of mechanical amoebocyte disruption have been proposed as a means of overcoming the variability of lysate potency (11, 12). The finding by Levin et al. (7) that four patients with fungemia due to Candida albicans had positive Limulus assays has also opened to question the specificity of the Limulus test for gram-negative bacterial endotoxin. This communication describes a slightly altered method of lysate preparation and relates our findings on the variability, sensitivity, and specificity of Limulus lysate.

MATERIALS AND METHODS

Preparation of Limulus lysate. Adult horseshoe crabs were purchased from the Marine Biological Laboratories, Woods Hole, Mass. Equipment used for lysate production and endotoxin assay was rendered sterile and pyrogen-free by washing and rinsing with pyrogen-free distilled water (Travenol Laboratories, Morton Grove, Ill.), steam sterilization at 121 C for 15 min, and dry-heat treatment at 170 C for 2 h. Sterile, disposable polystyrene pipettes (Falcon Plastics, Oxford, Calif.) were used throughout this investigation for transfer of lysate and test materials. The crab dorsal joint area was cleansed with a gauze pad moistened with 70% alcohol. A siliconized (Silicolad, Clay Adams Co., Parsippany, N. J.), pyrogen-free 14-gauge needle was inserted into the cardiac chamber by way of the dorsal junction of the cephalothorax of the crab (Fig. 1 and 2) as described by Reinhold and Fine (8). The hemolymph was allowed to flow directly into pyrogen-free siliconized 250-ml polypropylene centrifuge bottles containing 125 ml of 0.125% N-ethyl maleimide (Sigma Chemical Co., St. Louis, Mo.) dissolved in pyrogen-free 3% sodium chloride (Sigma), adjusted to pH 7.4 immediately before use with tri(hydroxymethyl)aminomethane buffer (Sigma), and warmed to 42 C. The amoebocytes, which are the only formed elements in Limulus hemolymph (Fig. 3), were sedimented by centrifugation at 50 x g for 10 min. The blue supernatant fluid was discarded, and the packed amoebocytes were transferred to pyrogen-free, siliconized 50-ml polypropylene centrifuge tubes and washed twice with pyrogen-free 3% sodium chloride to remove the N-ethyl maleimide. The cells were lysed by the addition of pyrogen-free distilled water (Travenol) at a 1:3 ratio of packed cells to water. The cell suspension was thoroughly mixed, with a Vortex
FIG. 1. Microscopy view of Limulus amoebocytes (×500 magnification). The dark, granular cells with light-staining nuclei are mature cells; cells with light-staining agranular cytoplasm and dark nuclei are immature forms.

Genie mixer, and allowed to stand at 4 C for 18 to 24 h. The cellular debris was then removed by centrifugation at 150 × g for 10 min, and the lysate was decanted. The lysate was stored in sterile pyrogen-free vials at −20 C or, for shorter periods of time, at 4 C.

Lysate batches were kept separate, based upon the individual crab donors, and numbered consecutively. Each lysate batch was subsequently tested for potency by assay with known levels of endotoxin, as described below.
**Performance of Limulus assay.** Endotoxin assays were conducted by adding a 0.1-ml sample of the material being tested to a 0.1-ml vol of *Limulus* lysate in 10 × 75-mm disposable glass test tubes (Corning Glass Works, Corning, N. Y.). Negative controls were performed by including a tube containing 0.2 ml of lysate only and a tube with 0.1 ml of the saline diluent used for the particular test material added to 0.1 ml of lysate. The reaction mixtures were incubated for 60 min at 37 C. The resultant reaction was observed and graded for degree and quality of gelation (Table 1 and Fig. 4).

**Endotoxin preparations.** Endotoxin preparations used in this study included Westphal phenol extracts of *Escherichia coli* O55:B5, *E. coli* O111:B4, *Salmonella enteritidis*, *Serratia marcescens*; Boivan trichloroacetic acid extracts of *E. coli* O55:B5, *E. coli* O111:B4, *S. enteritidis*, *S. marcescens*; and a tryptic digestion product of *S. marcescens*, all of which were purchased from Difco Laboratories, Detroit, Mich. In addition, a phenol extract of *Pseudomonas aeruginosa*, Verder and Evans group II, serotype 1369 (kindly supplied by J. A. Bass, North Texas State University) was prepared in our laboratory by the method of Selzer (10).

All endotoxin standard solutions were prepared by adding 10 mg of endotoxin to 10 ml of pyrogen-free 0.85% sodium chloride (Travenol), to yield a solution of 1 mg/ml concentration. This initial solution was diluted in pyrogen-free saline to give final concentrations of 10 ng/ml, 1 ng/ml, 500 pg/ml, 250 pg/ml, and 100 pg/ml for testing purposes. All endotoxin solutions were stored at 4 C in 16- by 125-mm sterile, disposable, polystyrene screw-cap test tubes (Falcon).

**Yeast.** Eleven species of both pathogenic and saprophytic yeasts were tested for endotoxin activity by use of the *Limulus* assay. These included six species of the genus *Candida*, *C. albicans*, *C. tropicalis*, *C. guillermontii*, *C. stellatoidea*, *C. krusei*, and *C. parapsilosis*; *Cryptococcus neoformans*, *Cryptococcus* species; *Torulopsis glutabrata*, *Rhodotorula* species, and *Saccharomyces cerevisiae*. All strains were grown in a medium consisting of yeast-nitrogen-base (Difco) with 0.5% glucose prepared by using pyrogen-free distilled water (Travenol). Each tube of medium was assayed for endotoxin activity and found to be negative, prior to its inoculation with the respective yeast. A sample of each culture was removed for *Limulus* assay after 24 h of incubation at 37 C, and again after 14 days of incubation at 25 C.

**RESULTS**

Thirty-six crabs were bled in our laboratory from September 7 to October 5, 1971, and 144 were bled from August 10 to September 28, 1972. Lysate batches were tested individually for potency by use of *E. coli* O55:B5 Boivan-extracted endotoxin (Difco). The lysate batches were then recorded as having a sensitivity capable of detecting a minimum of 1 ng/ml, between 1 and 10 ng/ml, or incapable of detecting as much as 10 ng/ml. Results of these lysate batch sensitivity determinations are shown in Table 2. The total lysate volume obtained and the mean volume per crab were calculated for both years. It should be noted that the volume of lysate per crab with a sensitivity of 1 ng/ml decreased more than threefold from 1971 to

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**Table 1. Grading of lysate gelation**

| Reaction   | Description                        |
|------------|------------------------------------|
| 4+         | Firm gel with considerable opacity |
| 3+         | Soft gel with moderate to considerable opacity |
| 2+         | Weak gel with slight to moderate opacity and adhesion of starch-like flocules to sides of tube when tube is slanted |
| 1+         | Very weak gel with slight opacity and with some starch-like flocules adhering to sides of tube |
| Negative   | No visible increase in viscosity or opacity |

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**Fig. 4. Close-up view of a negative Limulus test (top) and a strongly (4+) positive test (bottom).**
1972, whereas the volume per crab demonstrating a sensitivity of between 1 and 10 ng/ml remained approximately the same for both years. A marked variability was observed both years in regard to amounts of sensitive (1 ng/ml) lysate obtained from individual shipments of crabs (Fig. 5). This variability ranged from a high of 250 ml of sensitive lysate per dozen crabs on September 14, 1971, to periods in 1972 when no lysate of 1 ng/ml sensitivity was obtained.

Three batches of lysate, 1320 (1972), 3102 (1971), and 5602 (1972), were used to evaluate differences in sensitivities of separate lysate batches to the same endotoxin and differences in the sensitivity of the same lysate batch to a variety of endotoxins (Table 3). Differences among lysate batches were noted, with batch 1320 being most potent, batch 3102 being the least potent, and batch 5602 appearing to have an intermediate sensitivity. Some differences in activity among different endotoxins could be noted, and S. marcescens T and P. aeruginosa W demonstrated the least potency. However, a general degree of uniformity in terms of reactivity appeared to exist among all other endotoxin preparations tested.

All 11 yeast cultures had negative endotoxin assays from undiluted culture fluid and at a 1:5 dilution in saline at 24 h and again after 14 days of incubation.

**DISCUSSION**

The Limulus assay is a promising method for the detection of endotoxin from a variety of sources. This study has shown that as little as 100 pg/ml of certain endotoxins (Table 3) may be reliably detected with this method. The problem of lysate variability reported by Lindberg et al. (Fed. Proc., p. 791, 1972) is substantiated by the findings reported in this study. The causes of this variability are not easily identified. Great care was taken to maintain uniformity in the procedures used for lysate production with each shipment of crabs. The possibility that the animals were at times damaged in shipment could have been a contributing factor. However, the crabs were normally received and bled within 24 to 48 h of their departure from Woods Hole. The possibility also exists that some animals may have been bled previous to their acquisition for this study and, therefore, did not have a normal hemolymph composition. This could have been manifested as a predominance of immature amoebocytes yielding less clottable protein upon lysis. However, based on the report by Lindberg et al. who prepared lysate at the site of crab collection, the observed variability would seem to be biological variations among the amoebocytes, rather than factors involved in shipment or handling of the animals. This possibility is supported by the fact that there is a "season" from June or July through October in which amoebocyte lysate may be prepared, whereas at other times of the year, lysate preparation appears to be unsuccessful (Jacob Fine, personal communication, 1971). Newer methods of mechanical amoebocyte disruption (11, 12) may prove successful in overcoming this variability in lysate quality.

Levin and Bang (6) previously found that the rate and degree of gelation was directly dependent upon endotoxin concentration and independent of total protein content of lysate batches. However, a recent study (13) characterized three distinct protein fractions of lysate, one of which appeared to have enzymatic activity controlling the rate of gelation at a particular endotoxin concentration. Future investigations may elucidate methods whereby potency of lysate batches can be predicted by measurement of lysate composition. Presently, variability in potency of lysate batches is observed (Table 3) and is most readily documented by reaction of individual lysate batches with endotoxin. Therefore, when the Limulus assay is
used for quantification of endotoxin in various materials, individual lysate batches must be tested in advance by using a reference endotoxin standard to determine batch sensitivity. Knowledge of the batch sensitivity then allows for quantitation of endotoxin in terms of the concentration per milliliter of "endotoxin equivalents," based on the endotoxin standard. Variability among lysate batches may be minimized by pooling of individual lysate preparations of similar potency, followed by determination of the sensitivity of the lysate pool.

Endotoxins prepared by different methods from different organisms react with Limulus lysate at basically the same levels (Table 3). For instance, all of the endotoxin preparations, except for S. marcescens T, yielded a 4+ reaction at the 1 ng/ml level with lysate batch 1302. However, some differences in reactivity of different endotoxin preparations were apparent at concentrations below 1 ng/ml. It cannot be inferred from this study whether the differences in reactivity seen among various endotoxin preparations are greater than would be expected among different lots of the same endotoxin preparation. Therefore, any quantitative use of the Limulus assay should indicate the results in terms of endotoxin equivalents or endotoxin activity per milliliter based on a specific reference endotoxin.

The finding that yeasts fail to elicit a positive Limulus reaction support the observations of Cutler et al. (5) that cell wall extracts of C. albicans do not contain an endotoxin-like substance as measured by the Limulus test. These findings, combined with the previous observation (2) that dense cell suspensions of gram-positive bacteria failed to elicit a positive endotoxin reaction, emphasize the specificity of this test for bacterial endotoxin. When properly used and standardized, relative to specific endotoxin reference preparations, the Limulus assay appears to exhibit both sensitivity and specificity for bacterial endotoxin.

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### Table 3. Comparative activity of different endotoxins and different lysate batches

| Endotoxins (ng/ml) | 1320 | 3102 | 5602 |
|--------------------|------|------|------|
| E. coli O55:B5 W  | 4+   | 4+   | 4+   |
| E. coli O55:B5 B  | 4+   | 4+   | 4+   |
| E. coli O111:B4 W | 4+   | 4+   | 4+   |
| E. coli O111:B4 B | 4+   | 4+   | 4+   |
| S. enteritidis W  | 4+   | 4+   | 4+   |
| S. enteritidis B  | 4+   | 4+   | 4+   |
| S. marcescens W   | 4+   | 4+   | 4+   |
| S. marcescens B   | 4+   | 4+   | 4+   |
| S. marcescens T   | 4+   | 4+   | 4+   |
| P. aeruginosa W   | 4+   | 4+   | 4+   |

* W, Westpahl phenol extract; B, Boivan trichloroacetic acid extract; T, Tryptic digest; —, negative assay.

Note: The results are expressed as a reaction level on a 0-4 scale, where 4+ indicates the highest level of reaction.
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