Rapid detection of experimental *E. coli* endophthalmitis by the *Limulus* lysate test

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Experimental *E. coli* endophthalmitis was produced in rabbits. The *Limulus* lysate test was applied to aqueous and vitreous samples at various intervals after the intravitreal injection of *E. coli* organisms. Results indicated that this test is feasible using vitreous and aqueous samples. The *Limulus* test was positive for *E. coli* endotoxin within hours after infection, requiring only 1 hr to determine the presence of endotoxin after sampling. This test may have some value in the rapid diagnosis of gram-negative endophthalmitis.

Key words: *Limulus* assay, endophthalmitis, *E. coli*, rabbit model

The *Limulus* lysate test originally described by Levin and Bang has proved to be the most sensitive and specific test for gram-negative bacterial endotoxins available today. In addition, it is a simple test to perform and has been used for the detection of endotoxemia, gram-negative bacterial meningitis, and other conditions where the presence of gram-negative organisms is suspect.

The characteristics of this test led us to investigate its potential as a tool for the rapid detection of gram-negative endophthalmitis. The object of this report is to present our findings utilizing the *Limulus* test in experimental *Escherichia coli* endophthalmitis in rabbits.

Methods and materials

**Preparation of inoculum.** The gram-negative organism used in this investigation was *E. coli*. The inoculum, *E. coli* suspended in normal saline, was prepared by first growing the organism in broth (Callabs BHI with PABA and 0.1% agar), quantifying according to McFarland nephelometer standards, and then carrying out serial dilutions with normal saline until the desired concentration needed for injection was obtained. Concentration values were accurate to ±50% of the nominal nephelometer-predicted value, as determined by colony counts of cultures prepared from appropriate dilutions of inoculum.

Cultures were prepared from appropriate dilutions for verification of inoculum concentration.

**Infection of rabbit.** Four-pound New Zealand rabbits were used in this investigation. Rabbits were anesthetized by administering sodium thiopental–sodium pentobarbital intravenously through the marginal ear vein. Local anesthesia was effected by a 1.0 ml retrobulbar injection of 1% Lidocaine hydrochloride; proparacaine hydrochloride was applied topically.

After placement of lid speculum, 0.1 ml of the inoculum was injected into the vitreous cavity via the pars plana with the use of a 1 ml tuberculin syringe and 27-gauge needle, bevel-down.
Control (fellow) eyes were injected with sterile normal saline (0.1 ml) administered in the same manner.

**Paracentesis.** After one of the chosen time periods, either 0.5, 6, or 24 hr, samples were aspirated from the anterior chamber and vitreous of infected and control eyes. Anterior chamber taps yielded 0.1 to 0.2 ml samples with a 1 ml tuberculin syringe and a 27-gauge needle. Vitreous taps produced 0.2 to 0.4 cc samples with a 1 ml tuberculin syringe and a 22-gauge needle.

The anterior chamber was aspirated by inserting the needle bevel-up at the limbus, avoiding the iris. The vitreous sample was obtained by inserting the needle through the pars plana, 180 degrees from the site of inoculum injection. The needle was rotated and moved about in order to remove vitreous.

Samples were immediately centrifuged to remove any erythrocytes and debris, and the supernatant was removed and assayed immediately or refrigerated at 0° to 4° C to prevent the multiplication of any organisms present until the Limulus test could be performed.

All equipment and solutions were sterile and pyrogen-free. Syringes used for paracentesis contained a small amount (approximately 0.05 ml) of heparin as an anticoagulant. (Heparin does not affect the Limulus assay.)

**Limulus assay.** Limulus lysate was prepared and test-graded according to the method of Jorgensen and Smith and stored at —90° C. Performance of the test and grading of the lysate gelation were also performed according to their method. (See Table I) Samples of 0.1 ml were used for the test. Commercially prepared lyophilized Limulus lysate (Sigma Chemical Co., St. Louis, Mo.) was also tested, and results were comparable to our own lysate preparations. The amebocyte lysate was sensitive to 10 ng/ml E. coli lipopolysaccharide (Meyers and Chitjian, unpublished results). Limulus lysate activity was confirmed for each assay run by combining with 0.1 ml of serially diluted endotoxin solution. This reference endotoxin was prepared from a Westphal phenol extract of E. coli 0111:B4 (Difco Laboratories, Detroit, Mich.; 10 mg/ml concentrate).

Negative controls used for assay were a 0.2 ml sample of Limulus lysate only, a 0.2 ml sample consisting of 0.1 ml of normal saline and 0.1 ml of Limulus lysate, and a negative control of 0.2 ml of normal saline.

**Cultures.** Immediately prior to the performance of the Limulus test, a small portion of each vitreous and aqueous sample was plated on blood agar and incubated.

**Clinical examination.** During the acute experiments, the animal eyes were examined hourly and assessed for corneal haziness, red-reflex, hypopyon formation, and vitreous opacity. Similarly, after vitreous aspiration, the eyes were enucleated, the vitreous cavity exposed, and vitreous clarity assessed grossly.

**Results**

**Control eyes.** Fellow eyes injected with normal saline alone remained clinically quiet, and Limulus assays and cultures were always negative.

**Preliminary studies to determine inoculum size.** We attempted to determine the inoculum concentration that would produce negative Limulus test results at 0.5 hr (time 0 control), by running preliminary trials using inoculum concentrations ranging from 50 organisms (5 x 10^2/ml) to 50 million (5 x 10^9/ml). An inoculum concentration of 5 x 10^3/ml yielded a negative Limulus test at 0.5 hr, and one at 5 x 10^4/ml gave a positive Limulus test at 0.5 hr (for vitreous samples). Although negative at 0.5 hr, the eyes with 500 organisms were positive several hours after inoculation.

Data from eyes injected with 500 to 5000 organisms are presented in Table II at sampling times of 30 min, 6 hr, and 24 hr. An inoculum of 500 organisms gave negative Limulus test results at 30 min, but the Limulus assay was positive at the later times with incubation and multiplication of or-
Table II. *Limulus* and culture results—rabbit *E. coli* endophthalmitis (vitreous samples)*

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<th>No. of organisms injected</th>
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*For explanation of grading see Table I.

1) Indicates negative result.

2) Probable contamination.

organisms. These results were confirmed by the culture data. The data in Table I are for vitreous samples only. All anterior chamber samples were *Limulus*-negative, except for eyes injected with high concentrations of *E. coli* in pilot studies (in excess of the order of approximately $1.0 \times 10^7$ organisms/ml and sampled after 24 hr).

The results of the cultures prepared for the anterior and posterior chamber samples verified the results of the *Limulus* test. Except for one case (one eye inoculated with 500 organisms and assayed at 6 hr) where there was probably contamination of the culture plate, there was no positive culture in an eye with a negative *Limulus* test result. In this single instance, there was contamination of the 6 hr culture plate with another organism, probably the result of a break in laboratory technique.

Clinical examination. Severe anterior chamber reaction in the form of hypopyon and cloudiness of the aqueous was not found until the 24 hr examinations. This was not the case in earlier pilot studies where more massive doses of organisms were given in the inoculum and obvious infection was noted much sooner. There were a good red reflex and a clear vitreous in all of the 6 hr samples. The vitreous became very cloudy in the 24 hr samples in some cases.

Discussion

The basis of the *Limulus* assay is the coagulation or gelation of the blood corpuscles of the *Limulus* horseshoe crab upon exposure to endotoxins of various gram-negative organisms. Such coagulation does not occur under the influence of gram-positive organisms or their products. 3*4 The sensitivity and specificity of this test have been well studied in other organ systems, both in animals and in humans. 3*4 It has been shown on numerous occasions that false-positive results are not attained with gram-positive infections. Positive *Limulus* assays were obtained on vitreous and anterior chamber samples from rabbits with experimental *Pseudomonas aeruginosa* endophthalmitis and hypopyon keratitis (Brinkman, Meyers, and Chitjian, unpublished results). We also injected several rabbits intravitreally with *Staphylococcus aureus* and found negative vitreous and aqueous *Limulus* assays in such animals. It has also been shown that heparin, such as that used in many

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sampling techniques in the rabbit, does not impair the clotting ability of the lysate.\(^7\)

Causes of "false-positive" Limulus assays in other systems included pyrogen-contami-
nated glassware of diluent, synthetic poly-
nucleotides,\(^8\) certain isolated proteins,\(^8\) and serum from patients with hepatic dysfunction or undergoing radiation therapy.\(^9\)

"False-negative" assays have been noted in cases of variable potency of Limulus lysate,\(^2\) serum proteins which may bind endotoxin,\(^10\) low-potency endotoxin produced in exper-
imental strains of certain gram-negative bacteria,\(^3\) and extremes of pH.\(^10\)

We believe that intraocular assay tech-
niques, including the use of positive and negative controls, eliminate most of these possible sources.

The potential value of such a rapid assay in ocular infections is the rapid detection of endo-
phthalmitis caused by gram-negative organi-
isms and, consequently, the differentiation of this condition from either a gram-
positive endophthalmitis or benign aseptic postoperative inflammation. These results can be available within 1 hr or less of the sampling procedure, rather than in the 18 to 24 hr required to obtain culture evidence of infection.

The adaptability of this test to aqueous and vitreous samples presents no problem in the rabbit, and the small sample size obtained from the anterior chamber and vitreous taps is quite adequate for these studies.

Of importance is the finding that eyes which were infected but not clinically in-
famed, with no hypopyon, were Limulus-
positive from vitreous taps, despite the fact that the anterior chamber taps were always Limulus-negative. This confirms the findings of Forster,\(^11\) who found vitreous taps to be more productive of culture-proven disease than were anterior chamber taps. This result also emphasizes the sensitivity of the Limulus assays, in that very early infection may result in positive assays.

There were no false-positives from the control eyes injected with saline, and there were no positive cultures in any eye which had a negative Limulus test result, except in one eye which was probably due to contami-
nation. More importantly, there was not a single case in which gram-negative endoph-
thalmitis could be detected clinically but which had a negative Limulus assay. Although the sensitivity of the lysate used was 10 ng/ml lipopolysaccharide standard as compared to 0.1 ng/ml of Levin and Bang.\(^1\)\(^7\)

The fact that the assay as employed in this study was more than adequate to detect early infection, in addition to being commercially available, suggests its clinical usefulness.

We believe that this test is a potentially useful tool which may become more important if the use of intravitreal antibiotics and vitrectomy are subsequently shown to be of value in the management of endophthalmitis. It would be extremely valuable to be able to determine the presence or absence of gram-negative infection in the operating room by the use of this test, in order to more effect-
tively select antibiotics for intravitreal use and to determine the value of immediate vit-
rectomy on the basis of such results.

Clinical evaluation of this technique is cur-
rently underway in our laboratories.

REFERENCES


3. Jorgensen, J. H., and Smith, R. F.: Preparation, sensitivity, and specificity of Limulus lysate for en-


5. Levin, J., Poore, T. E., Young, N. S., et al.: Gram-


7. Levin, J., and Bang, F. B.: Clottable protein in Limulus: its localization and kinetics of its coagula-


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