Rapid Diagnosis of Gram-Negative Bacterial Meningitis by the *Limulus* Endotoxin Assay

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The *Limulus* amoebocyte lysate endotoxin assay was evaluated as a method for rapid diagnosis of acute bacterial meningitis in a series of 305 patients. The results of *Limulus* assays on cerebrospinal fluid (CSF) samples from these patients were compared with the results for each patient of routine bacterial cultures and Gram stains. Positive *Limulus* tests were obtained on initial CSF specimens from 84% of patients with culture-proven bacterial meningitis, including all patients with meningitis due to gram-negative organisms. Initial Gram-stained smears revealed the presence of organisms in 68% of the patients. One patient with pneumococcal meningitis had a weakly positive *Limulus* assay, whereas patients with meningitis due to other gram-positive organisms, those with aseptic meningitis, or patients without meningitis had negative CSF *Limulus* tests. The *Limulus* assay also demonstrated the persistence of endotoxin in the CSF of certain patients during antibiotic therapy, especially patients with *Haemophilus influenzae* meningitis. The *Limulus* test proved to be a rapid, reliable indicator of the presence of gram-negative organisms in the CSF of patients suspected of acute bacterial meningitis.

The mortality associated with acute bacterial meningitis can be greatly reduced if appropriate antibacterial therapy is initiated promptly (17). The usual laboratory diagnostic methods to differentiate bacterial from viral or other agents of meningitis are a culture of cerebrospinal fluid (CSF) for bacterial pathogens, direct microscopic examination of CSF for bacteria and leukocytes, and measurement of CSF glucose and protein concentrations. If treatment is to be successful, antibacterial therapy must be initiated before culture results become available (17). However, results of microscopical, chemical, and hematological examinations of CSF may be inconclusive or totally misleading, especially in early bacterial meningitis (7). The examination of Gram-stained smears of CSF for bacteria has been shown to yield reliable information regarding the etiological agent involved in only 60 to 80% of cases, even in the most experienced hands (2, 25). Therefore, more precise, rapid methods for the early detection of meningitis are urgently needed.

Current rapid methods for the diagnosis of bacterial meningitis are mainly immunological techniques for the demonstration of bacterial antigens in CSF. Counter-immunoelectrophoresis (CIE) has been used for the detection of meningococcal, *Haemophilus*, and pneumococcal antigens in the CSF of patients with menin-

gitis (3, 5, 10, 11, 18, 20). Latex slide agglutination techniques for the detection of these antigens (22, 26) also appear promising. However, both of these techniques require high-titer-specific antisera that may be difficult to obtain commercially. The determination of the CSF lactate concentration (8) may also help to distinguish bacterial from viral or fungal meningitis.

The *Limulus* in vitro endotoxin assay has been used previously for the detection of endotoxin in blood (6, 15, 24) and urine (12, 13) and as a method for pyrogen testing of injectable pharmaceutical products (16, 19). This test is currently the most sensitive method available for the detection of endotoxin. The results of initial studies concerning the use of the *Limulus* assay for the rapid detection of gram-negative bacteria in CSF appear very encouraging (1, 21, 23). This study describes our recent experience using this technique for the rapid diagnosis of gram-negative bacterial meningitis.

MATERIALS AND METHODS

Patient group. Adult and pediatric patients of the Bexar County Hospitals, on whom a lumbar puncture was performed because of suspected meningitis, were included in this study. On each CSF specimen, a Gram stain and culture, as well as *Limulus* assay, were performed.

Performance of *Limulus* assays. CSF samples for *Limulus* testing were collected in sterile, pyrogen-

free plastic or glass test tubes. Two different *Limulus* amoebocyte lysate preparations were used in this study, depending upon availability. Amoebocyte lysate was prepared in our laboratory by methods previously described (14), and a commercial lysate preparation, Difco Pyrotest (generously supplied for this study by Difco Laboratories, Detroit, Mich.), was also used. The sensitivities of individual lysate preparations were determined as previously described (14) by testing with *Escherichia coli* O124:B4 lipopolysaccharide, also supplied by Difco Laboratories. *Limulus* lysate prepared in our laboratory could detect as little as 0.5 ng of lipopolysaccharide per ml, whereas Pyrotest could detect as little as 0.06 ng/ml. Individual *Limulus* test reactions on patient CSF specimens were graded for reactivity with a scheme previously described (14). To perform a *Limulus* test on a CSF specimen with our lysate, 0.1 ml of the CSF specimen was added to a sterile, pyrogen-free glass test tube (10 by 75 mm) to which 0.1 ml of *Limulus* lysate had been added. For specimens examined using Pyrotest, a 0.2-ml sample of CSF was added directly to the lyophilized *Limulus* amoebocyte lysate in a single test vial. Both positive and negative controls were run with each set of assays. The positive control consisted of the addition of an appropriate volume of a 1-ng/ml stock solution of *E. coli* lipopolysaccharide to an additional tube of the particular lysate preparation. Addition of pyrogen-free saline or water to another tube of lysate constituted a negative control. All *Limulus* assays were incubated at 37°C for 70 min in a bacteriological incubator. After incubation, each tube was examined for the presence of a gel or a definite increase in viscosity and turbidity compared with the negative control tube (14). Both a strongly positive (4+) reaction in the positive control tube and a negative (water-like) reaction in the negative control tube were required for a valid assay set.

Occasionally, the volume of the CSF specimen was insufficient to be processed in the above manner. In such instances, a portion of sterile, pyrogen-free water (Travenol Laboratories, Morton Grove, Ill.) was added to the existing amount of CSF to yield a final volume of at least 0.1 ml. The diluted specimen was then assayed in the manner described above, except that an additional 0.1 ml of water was added to the Pyrotest vials to bring the final test volume to 0.2 ml.

RESULTS

CSF specimens from a total of 305 patients suspected of bacterial meningitis were examined by the *Limulus* test as well as by standard bacterial culture and Gram stain. A total of 74 patients had culture-proven acute bacterial meningitis (Table 1). These included 61 patients with meningitis due to gram-negative organisms and 13 patients from whom gram-positive organisms were isolated. Only 1 patient had tuberculous meningitis, whereas the remaining 230 patients either did not have meningitis or were diagnosed as having aseptic meningitis. All of the initial CSF specimens from the 61 patients with gram-negative bacterial meningitis yielded positive *Limulus* assays (Table 1).

Limulus tests were negative from all but 2 patients whose CSFs were culture-negative, from the patient with tuberculous meningitis, and from 12 of 13 patients with gram-positive bacterial meningitis. The one exception in the latter group was a weakly positive (1+) reaction encountered with one CSF sample from a patient with pneumococcal meningitis. The two patients whose CSFs yielded apparent false-positive *Limulus* assays had not received prior antibiotic therapy.

Gram stains were positive on CSFs from 72% (44/61) of patients with gram-negative bacterial meningitis and only 46% (6/13) of patients with gram-positive meningitis. Gram stains were negative on all CSFs that were culture negative and on the CSF from the patient with tuberculous meningitis.

A total of 22 patients were found to have persistent endotoxin in subsequent CSF specimens while on antibiotic therapy (Table 2). Of these patients, 13 had *Haemophilus influenzae* isolated from their initial CSF specimens. Among these, cultures of CSF from repeat lumbar punctures were negative in 13 instances, and the Gram stain was only positive in 1 case, although *Limulus* assays continued to be positive in all 13 patients. In contrast, all of the nine patients with non-*Haemophilus* gram-negative meningitis and persistent CSF endotoxin had positive CSF cultures. Only 1 of the 9 had posi-

TABLE 1. Results of initial CSF examinations on 305 patients suspected of bacterial meningitis

Organism isolated	No. of patients	<i>Limulus</i> positive	Gram stain positive
<i>H. influenzae</i>	38	38	30
<i>Neisseria meningitidis</i>	6	6	5
<i>E. coli</i>	6	6	3
<i>Klebsiella pneumoniae</i>	2	2	0
<i>Alcaligenes faecalis</i>	1	1	1
<i>P. aeruginosa</i>	4	4	2
<i>Flavobacterium meningosepticum</i>	1	1	1
<i>Acinetobacter calcoaceticus</i> var. <i>anitratus</i>	1	1	0
<i>A. calcoaceticus</i> var. <i>lwoffi</i>	1	1	1
<i>Citrobacter diversus</i>	1	1	1
Group B <i>Streptococcus</i>	4	0	2
<i>Streptococcus pneumoniae</i>	6	1 ^a	3
<i>Staphylococcus aureus</i>	3	0	1
<i>Mycobacterium tuberculosis</i>	1	0	0
Aseptic or no meningitis	230	2	0

^a Graded as 1+ reaction; see text.

TABLE 2. *Patients with persistent CSF endotoxin during antibiotic therapy*

Organism isolated	No. of patients	<i>Limulus</i> positive	Repeat culture positive	Repeat gram stain positive
<i>H. influenzae</i>	13	13	1	1
<i>E. coli</i>	3	3	3	1
<i>Neisseria meningitidis</i>	1	1	1	0
<i>Klebsiella pneumoniae</i>	1	1	1	0
<i>Flavobacterium meningosepticum</i>	1	1	1	0
<i>P. aeruginosa</i>	2	2	2	0
<i>Acinetobacter calcoaceticus</i> var. <i>anitratus</i>	1	1	1	0

tive Gram stain from the follow-up CSF specimen.

The time relationship of positive CSF endotoxin assays from patients with *H. influenzae* meningitis was also examined. With CSF endotoxin assays performed after the initiation of therapy in patients with *H. influenzae* meningitis, positive *Limulus* assays were found as follows: at 24 h, six of nine patients; at 48 h, five of seven patients; at 72 h, one of one patient; and at >72 h, two of six patients. All specimens were culture negative for *H. influenzae*. As can be seen from these data, it was not unusual for patients with sterile repeat CSF specimens to have a persistence of endotoxin 24 to 72 h after the initiation of antibiotic therapy, with one patient demonstrating persistent CSF endotoxin for 9 days.

Two specific cases among this group were studied in greater detail. Table 3 indicates the laboratory results on patient J.M., a 7-month-old child with *H. influenzae* meningitis who had persistent CSF endotoxin for at least 9 days after the initiation of antibiotic therapy, although the CSF culture was negative after 48 h of therapy with chloramphenicol. Patient J.M. recovered without apparent neurological deficits.

Patient R.P. was a 51-year-old male who developed meningitis with *Pseudomonas aeruginosa* after a neurosurgical procedure (Table 4). The initial CSF *Limulus* assay was strongly positive. A CSF specimen obtained 48 h after the initiation of therapy was both sterile and *Limulus* test negative. However, subsequent samples obtained between 72 h and 11 days after the initiation of therapy demonstrated the growth of a few colonies of *Pseudomonas*, but persistently negative *Limulus* tests. A repeat CSF specimen obtained on day 11 of therapy

was again strongly *Limulus* test positive. The patient expired shortly after the last CSF sample was obtained.

DISCUSSION

A rapid, reliable laboratory method for the diagnosis of bacterial meningitis would allow a prompt initiation of antimicrobial chemotherapy. Rapid immunological methods, such as CIE, may detect the presence of antigens of certain of the pathogens most often encountered in bacterial meningitis. However, organisms not reactive with the specific test battery of antisera will be missed by this technique. The *Limulus* endotoxin assay is a rapid and reliable method for the demonstration of a variety of bacterial antigens in certain body fluids, including CSF.

In the present study, *Limulus* assays were easily interpretable and, when positive, usually could be read after only 20 to 30 min of incubation. The commercial lysate preparation (Pyrotest) was found to be superior in sensitivity to amoebocyte lysates prepared in our laboratory. When more than 100 assays were performed in parallel, no discrepancies were found between results obtained using the two lysate preparations, although positive reactions were more definite and occurred sooner with the Pyrotest. False-negative (or -positive) results were not observed in those instances in which sterile wa-

TABLE 3. *Persistence of CSF endotoxin for 9 days in spite of negative CSF cultures in patient J.M.*

Day	Culture result	Gram stain	<i>Limulus</i> assay result
0	<i>H. influenzae</i>	GNR ^a	4+
2	No growth	NBS ^b	2+
4	No growth	NBS	4+
6	No growth	NBS	1+
9	No growth	NBS	1+

^a GNR, Gram-negative rods.

^b NBS, No bacteria seen.

TABLE 4. *Persistence of organisms in CSF cultures for 11 days in patient R.P. although most Limulus assays were negative*

Day	Culture result	Gram stain result	<i>Limulus</i> assay result
0	<i>P. aeruginosa</i>	GNR ^a	4+
2	No growth	NBS ^b	Neg. ^c
3	<i>P. aeruginosa</i>	NBS	Neg.
6	<i>P. aeruginosa</i>	NBS	Neg.
7	<i>P. aeruginosa</i>	NBS	Neg.
11	<i>P. aeruginosa</i>	NBS	4+

^a GNR, Gram-negative rods.

^b NBS, No bacteria seen.

^c Neg., Negative.

ter was added to supplement the volume of CSF specimens that were insufficient in volume for assay. The degree of gelation did not appear to be diminished by dilution of these specimens, although the full 70-min incubation was observed before examining the lysate tubes.

Table 5 shows the total accumulative data from the present investigation and previous similar studies (1, 4, 18, 21, 23, 29) evaluating the *Limulus* assay for the diagnosis of bacterial meningitis. These combined data represent a total of 393 patients with culture-proven acute bacterial meningitis, including 321 cases due to gram-negative organisms. If data from all six studies are combined, 92% of all patients with culture-proven, gram-negative bacterial meningitis were rapidly detected by the *Limulus* test. For purposes of comparison in the present study, only 68% of cases were correctly recognized by Gram stain of the CSF. If all cases of culture-proven bacterial meningitis in these studies are considered, the *Limulus* assay detected 75% of cases. The 25% negative rate is due almost entirely to the lack of detection of gram-positive organisms. Only the study by McCracken and Sarff (18), involving neonatal meningitis cases, has reported a significant number of false-negative *Limulus* tests on patients with documented meningitis due to gram-negative organisms. Their finding of endotoxin in only 71% of CSF samples from infants with gram-negative bacterial meningitis is in contrast to the findings of the current study and those previously (1, 4, 21, 23, 29).

The main predictive value of the *Limulus* assay lies in its low percentage of false-positive reactions (<1%). Any patient with a positive CSF *Limulus* test should therefore be consid-

ered to have gram-negative bacterial meningitis (1). However, a negative assay does not rule out the possibility of meningitis due to gram-positive organisms, e.g., infections caused by *S. pneumoniae* or group B *Streptococcus*.

If the previous studies using CIE (3, 5, 10, 11, 20, 27) and those using the *Limulus* test (1, 4, 18, 21, 23, 29) are reviewed, it becomes apparent that the overall abilities of the two procedures to detect patients with meningitis are quite similar, although for different reasons. CIE suffers from false-negative reactions either because of too little antigen in the CSF to be detectable or perhaps because of poor migration of some antigens, such as pneumococcal polysaccharide. The *Limulus* assay is wholly unable to detect antigens of gram-positive organisms, but is very sensitive to the presence of cell wall antigens of gram-negative bacteria. A recent study (9) demonstrated that concentrations of bacteria in CSF of patients with meningitis ranged from 4.5×10^3 to 3×10^8 organisms per ml. Previous investigations (12, 28) have shown that the threshold of detectability by the *Limulus* assay based on the detection of cell-bound endotoxin is approximately 10^2 to 10^3 organisms per ml. Therefore, the sensitivity of the *Limulus* test for the detection of gram-negative organisms in CSF is easily explained.

There are not yet sufficient data available to comment on the relative merits of CIE or the *Limulus* assay for the diagnosis of patients with partially or inappropriately treated meningitis. However, the persistence of antigens in the CSF for 24 to 48 h after the initiation of therapy has been reported by using both methods (3, 10, 18, 21, 23). The prognostic value of CIE or *Limulus* assay on follow-up CSF specimens during therapy for meningitis has also not yet been fully clarified. It appears that antigens may persist in the CSF for 48 h or longer, despite sterile CSF cultures. In the current study, the implication of persistent CSF endotoxin differed, depending upon the causative organisms. In non-*H. influenzae* meningitis, the persistence of endotoxin suggested that therapy was inadequate because the CSF had not been sterilized. However, with *H. influenzae* meningitis, endotoxin persisted in the CSF of some patients for 72 h or more, although viable organisms could no longer be recovered.

Prior clinical applications of the *Limulus* assay have included the demonstration of endotoxin in plasma as an indicator of gram-negative septicemia (15, 24) and a measurement of endotoxin in urine for the detection of bacteriuria (12, 13). The examination of plasma for endotoxin has not been uniformly successful with all

TABLE 5. Accumulative data from six evaluations of the *Limulus* assay for diagnosis of bacterial meningitis

Study	No. of patients with positive CSF cultures	No. of patients with gram-negative organisms in CSF	No. of patients with positive CSF <i>Limulus</i> assays
Berman et al. (1)	107	86	86
Dyson and Cassidy (4)	10	6	6
McCracken and Sarff (17, 18)	94	84	60 ^a
Nachum et al. (21)	43	38	38
Ross et al. (23)	51	38	37
Tuazon et al. (29)	14	8	8
Present study	74	61	61

^a Number of patients derived from author's statement that "endotoxin was detectable in only 71% of CSF samples obtained on the initial lumbar tap of infants with Gram negative bacterial meningitis."

investigators (6, 28). This is due at least in part to the technical problems associated with the preliminary extraction procedure with chloroform or other similar treatments that are required when plasma proteins are involved. Fluids, such as CSF or urine, do not require these extraction procedures and may therefore be examined by the *Limulus* assay with much greater facility.

In the present study, 100% of patients with gram-negative meningitis were easily identified within 1.5 h by use of the *Limulus* assay, and in many cases within 30 min. Results of *Limulus* assays were more easily interpreted and more reliable than were Gram stain results. The primary disadvantage of this technique is the inability to detect gram-positive organisms, which lack endotoxin. Therefore, the usefulness of this test lies in the examination of CSF from patients with suspected bacterial meningitis in which gram-negative organisms are the predominant agents. These include children, patients who are immunodeficient or on immunosuppressive medications, and patients who have received surgical procedures or traumatic injuries of the central nervous system.

The ability to obtain high-quality *Limulus* amoebocyte lysate commercially now makes this test a feasible adjunct to current diagnostic procedures for bacterial meningitis. However, use of this test in a routine hospital diagnostic laboratory requires that the amoebocyte lysate be licensed by the Food and Drug Administration as an in vitro diagnostic product. This licensure has unfortunately not yet been obtained. Therefore, *Limulus* assay of CSF or any body fluid for the diagnosis of bacterial infection continues to be a research technique that requires in most locales the informed consent of the patient.

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