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# Detection of Free Endotoxin in Cerebrospinal Fluid by the *Limulus* Lysate Test

ROBERT S. MUNFORD,[1,2]* CATHERINE L. HALL,[1,2] AND LEE GRIMM[3]

*Departments of Internal Medicine,[1] Microbiology,[2] and Pediatrics,[3] the University of Texas Health Science Center, Southwestern Medical School, Dallas, Texas 75235*

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**We used a rabbit model of *Escherichia coli* meningitis to study the basis for positive *Limulus* lysate tests in infected cerebrospinal fluid. The results indicated that positive *Limulus* tests are due to endotoxins in cerebrospinal fluid and not to leukocyte proteases or other possible activators of the *Limulus* clotting system. The results also suggest that bacteria-free endotoxin may be present in localized gram-negative bacterial infections.**

The *Limulus* lysate test is an in vitro clotting assay that has been widely used to detect gram-negative bacterial lipopolysaccharides (LPS; endotoxins). Although the assay is very sensitive, its specificity for LPS remains in some doubt, particularly when the test is applied to specimens that contain proteases that may activate the *Limulus* clotting cascade (3). The best available evidence for the specificity of the *Limulus* test is provided by studies of cerebrospinal fluid (CSF) from patients with meningitis; several investigators have found excellent correlation between the results of *Limulus* tests on CSF and the presence or absence of gram-negative bacteria in the same specimens (1, 5, 12, 18). On the other hand, the basis for the positive tests is not known. It is not certain that the positive tests are produced by LPS per se, and not by protease(s) or other factors released into the CSF by LPS-stimulated leukocytes (4, 11, 15, 16, 18).

To examine the basis for positive *Limulus* tests in infected fluid, we assayed samples of CSF that were obtained from rabbits with experimental meningitis. New Zealand white male rabbits (2 to 3 kg) were prepared for cisternal inoculation as described by Schaad et al. (14). *Escherichia coli* O7K1 cells were grown to mid-log phase in proteose peptone-beef extract broth (10), washed once with normal saline, and adjusted to a concentration of $7 \times 10^5$ organisms per ml. Fifteen to 18 h after the intracisternal injection of 1.0 ml of inoculum, cisternal puncture was repeated to obtain purulent CSF. The fluid was immediately placed in an ice bath. The number of viable organisms in the CSF was determined by plate cultures of serial dilutions of CSF in normal saline; the serotype (O7) of the infecting organisms was confirmed for three colonies from each culture. The number of leukocytes was measured with a hemacytometer. The CSF was centrifuged (2,000 × *g*, 10 min, 4°C), and the resulting supernatant was filtered (0.45 μm) to obtain leukocyte- and bacteria-free CSF; plate cultures of 100 μl of CSF were negative. Portions of the filtered CSF were then frozen (−70°C). Quantitative *Limulus* lysate assay and solid-phase radioimmunoassay (RIA) for O7 antigen were performed as previously described (6, 7); LPS extracted by the hot phenol-water method (19) from *E. coli* O7K1 was used as the standard for both assays. Samples for the *Limulus* assay were diluted 10- to 1,000-fold in pyrogen-free water (Travenol Labs, Deerfield, Ill.) to reach the assay range (0.2 to 2 ng/ml), whereas samples for RIA were diluted 1:5 in phosphate-buffered saline-Tween 20 (7). Each assay result is the mean of three or four determinations. Linear regression analysis was performed with a Radio Shack model EC-4004 calculator.

Quantitative cultures of CSF samples from 10 infected rabbits revealed from $4 \times 10^2$ to $1 \times 10^9$ CFU/ml. Bacteria-free LPS were detectable in every sample by *Limulus* assay (minimal detectable concentration, 0.0002 μg/ml) and in 8 of the 10 samples by RIA (minimal detectable concentration, 0.008 μg/ml). There was good correlation between the numbers of CFU and the amounts of LPS measured in bacteria-free CSF by the *Limulus* test (Fig. 1, panel A) and the RIA (Fig. 1, panel B). Moreover, although each test for LPS gave negative results on samples of uninfected CSF, the correlation between the two assays on samples of infected CSF was excellent (Fig. 1, panel C). There was poor correlation between the results of the assays for LPS and the numbers of leukocytes found in the samples of infected CSF (Fig. 1, panel D).

The *Limulus* test was also performed on bacteria-free CSF before and after *E. coli* O7 LPS was removed from the CSF. Homologous immunoadsorption by anti-LPS immunoglobulin G reduced the amount of LPS that was detected by the *Limulus* assay by ca. 20-fold (Table 1).

The results indicate that the *Limulus* lysate test on leukocyte- and bacteria-free CSF is specific for LPS. This conclusion is supported by (i) the direct correlation between the results of this test and the results of an independent assay for LPS and (ii) the ability of adsorption with anti-LPS antibodies to reduce the activity of the samples in the *Limulus* assay.

Previous workers have found good correlation between the results of *Limulus* tests and the results of other bioassays for endotoxin activity (2, 17). To our knowledge, our data provide the first comparison of the *Limulus* assay with a quantitative immunoassay with test samples of an infected body fluid. The results of the O antigen RIA were ca. 10-fold higher than the results of the *Limulus* test, regardless of the amount of LPS in the sample (Fig. 1). In contrast, when purified LPS were added to CSF, essentially identical amounts of LPS were measured by the two assays (data not shown). The explanation for these findings is uncertain. It is possible that the discrepancy between the results of the two assays, when used to test meningitis samples, relates to the different physical states of the purified LPS standard (pre-

* Corresponding author.

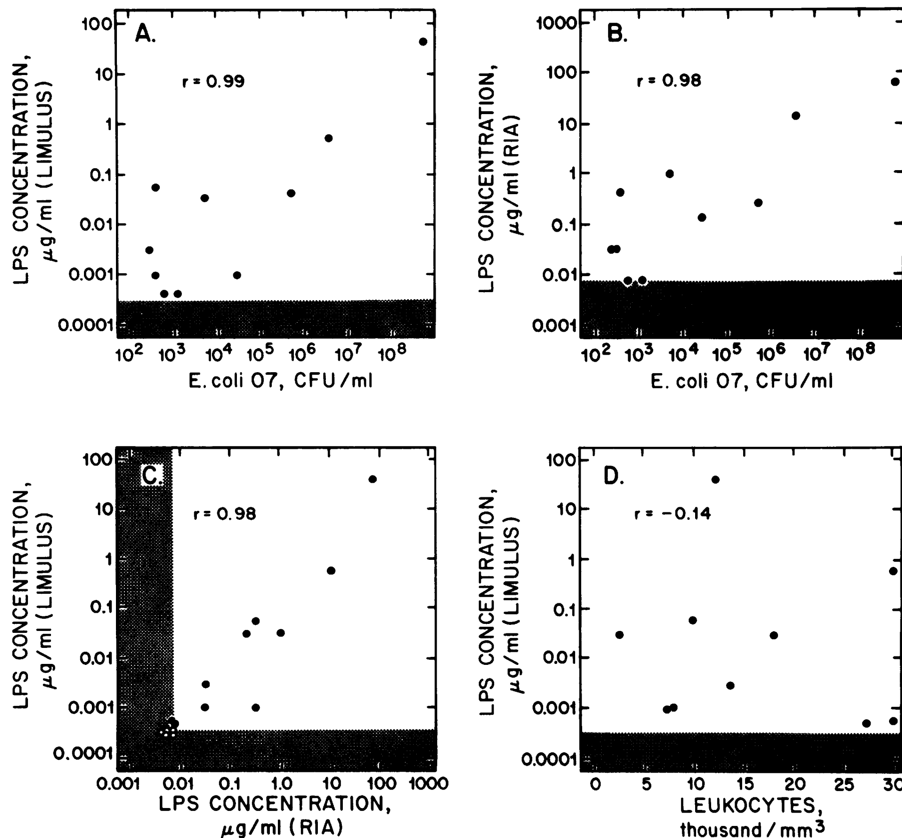


FIG. 1. Relationship between LPS concentration as measured by *Limulus* assay and numbers of viable bacteria (panel A), LPS concentration as measured by RIA and numbers of viable bacteria (panel B), LPS concentration as measured by *Limulus* and RIA (panel C), and LPS concentration as measured by *Limulus* assay and total leukocyte counts in infected CSF (panel D). Each closed circle represents CSF from a single rabbit. The open circles in panel C represent samples of uninfected CSF. The shaded areas indicate the detection limits of the assays.

pared by phenol extraction) and the native LPS in the meningitis CSF samples (probably present in fragments of bacterial outer membrane, so that the active lipid A moiety is relatively hidden from the *Limulus* enzyme [8, 9, 13]). Alternatively, components of the pus may alter the native LPS, or incomplete LPS (free O antigen) may be present in the infected samples. In any case, our results indicate that assays for different LPS regions may give quantitatively different results, even when the same preparation of purified LPS is used to standardize both assays.

The existence of bacteria-free LPS has not been unambiguously demonstrated during infection in vivo (9, 13). Although we cannot entirely exclude the possibility that the bacteria-free LPS were released from the bacteria while handling the specimens in the laboratory, our results are consistent with the conclusion that LPS are shed from bacteria into infected CSF. We thus provide support for the idea that bacteria-free LPS, released in vivo, may have biological effects that are independent of the LPS in the bacterial cell wall.

TABLE 1. Limulus lysate tests on bacteria-free CSF[a]

| Immunoadsorption with immunoglobulin G antibodies to: | LPS concentration (µg/ml) | |
|---|---|---|
| | Expt 1 | Expt 2 |
| None (unadsorbed) | 0.68 | 39 |
| *E. coli* O6K13 (heterologous) | 0.59 | 45 |
| *E. coli* O7K1 (homologous) | 0.01 | 2.1 |

[a] Cowan strain *Staphylococcus aureus* were coated with rabbit immunoglobulin G to *E. coli* O6 or *E. coli* O7 as described previously (8). The coated staphylococci were washed three times with 0.04 M potassium phosphate (pH 7.2) prepared in pyrogen-free water. Bacteria-free CSF samples (50 µl) and samples of packed staphylococcal cells (50 µl) were added to 0.45 ml of 0.04 M potassium phosphate and incubated, with occasional mixing, for 15 h at 4°C. Control samples were incubated without added staphylococci in the same volume of 0.04 M potassium phosphate. After centrifugation to pellet the staphylococci, the supernatants were diluted in pyrogen-free water and tested for activity in the *Limulus* assay. The results of two separate experiments (different rabbits) are shown; each result is the mean of three measurements. Standard deviations were less than 10% of the means.

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