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Response of Several *Limulus* Amoebocyte Lysates to Native Endotoxin Present in Gonococcal and Nongonococcal Urethral Exudates from Human Males

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Three *Limulus* amoebocyte lysate (LAL) preparations obtained from three different suppliers were comparatively evaluated for sensitivity to native endotoxin contained in urethral exudates from 28 men with gonococcal urethritis and 16 men with nongonococcal urethritis. One LAL preparation was not extracted with organic solvents during manufacture, whereas the other two were extracted with chloroform. All three LAL preparations had equivalent sensitivities (0.06 ng/ml) to an established reference endotoxin standard (EC-2), but significant differences in sensitivities were found among the different LAL preparations when testing clinical specimens. Dilution breakpoints of urethral samples for maximum sensitivity and specificity ranged from 1:400 to 1:1,600, depending on the LAL preparation. The nonextracted lysate was significantly more sensitive to the presence of endotoxin in gonococcal exudates than the other two preparations ($P < 0.001$) but not significantly different from one LAL preparation ($P > 0.05$) in detecting endotoxin in nongonococcal exudates. An additional 116 men, 61 with culture-proven gonococcal urethritis and 55 with nongonococcal urethritis, were evaluated with three lots of nonextracted lysate with sensitivities ranging from 0.04 to 0.06 ng/ml, reference endotoxin EC-2. At a dilution breakpoint of 1:1,600, the sensitivity of the LAL test was 100%, and the specificity was 96%.

In a recent report (9), we demonstrated the potential utility of the *Limulus* amoebocyte lysate (LAL) assay as a rapid, reliable, sensitive, and specific test for the presumptive diagnosis of gonococcal urethritis in men. In that report and in another using the microdilution LAL technique (7), we used lysate preparations that were chloroform extracted by the manufacturer to remove inhibitors of gel formation for improved sensitivity.

Recently, while testing gonococcal and nongonococcal urethral samples, we found that a commercially available lysate not extracted with organic solvents appeared to be more sensitive than the chloroform-extracted lysate in detecting native endotoxin in urethral exudate even though the lysate preparations were similar in sensitivities to an established reference endotoxin standard. The purposes of this study were to evaluate comparatively both organic solvent-extracted and nonextracted LAL preparations of the same sensitivities for the quantitative determination of native endotoxin in gonococcal and nongonococcal urethral exudates and to evaluate the nonextracted LAL preparation in the initial evaluation of exudative urethritis in men.

MATERIALS AND METHODS

Study population. Men with uncomplicated urethritis seen at the Columbus Health Department Venereal Disease Clinic formed the study population. Forty-four men were used in the comparative studies of chloroform-extracted and nonextracted LAL preparations, and 116 additional men were evaluated by the nonextracted lysate. These patients had sought treatment because of urethral discharge or dysuria or both and were selected on a random basis; only patients with a purulent urethral discharge or a discharge obtained after urethral massage were used. Patients receiving antibiotics within 10 days of presentation were excluded.

Diagnostic procedures. The routine diagnostic methods used in the clinic for the evaluation of exudative urethritis have been previously described (9). Before collection of a urethral sample for culture of *Neisseria gonorrhoeae* and Gram staining, a small amount of urethral exudate (0.025 to 0.05 ml) was collected from the urethral meatus by gentle aspiration with a tuberculin syringe (without needle) and transferred to a pyrogen-free plastic test tube containing 1 ml of pyrogen-free water (Travenol Laboratories, Deerfield, Ill.). The samples were then adjusted to a dilution of 1:50 with water and frozen at −20°C before being tested by the LAL assay.

Examination of Gram-stained smears of urethral exudate and identification of isolates of *N. gonor-*

TABLE 1. *Comparative LAL test results for three LAL preparations at various dilutions of 28 gonococcal and 16 nongonococcal urethral samples*

LAL prepn[a]	Diagnosis[b]	% Positive at dilution 1:											
		50	100	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200	102,400
MK	GU	100	100	100	100	100	100	96	86	64	57	46	39
	NGU	94	75	38	19	6	0						
CC	GU	100	100	100	100	100	96	89	71	64	46	39	29
	NGU	75	63	31	6	0							
MA	GU	100	100	100	100	96	71	57	39	32	18	4	0
	NGU	56	25	0									

[a] Minimum sensitivity of each LAL preparation = 0.06 ng/ml, standard reference endotoxin EC-2.
[b] GU, Gonococcal urethritis; NGU, nongonococcal urethritis.

TABLE 2. *Interlysate statistical comparisons of sensitivities to exudate from men with gonococcal and nongonococcal urethritis*

Lysate comparisons	*P* value[a]	
	Gonococcal urethritis	Nongonococcal urethritis
MK vs CC	<0.001	NS
MK vs MA	<0.001	<0.01
CC vs MA	<0.001	<0.01

[a] Single-tailed paired *t* test. NS, Not significant ($P > 0.05$).

rhoeae were performed as previously described (9). Cultures for viruses or chlamydiae were not done.

LAL preparations. Three LAL preparations from three manufacturers were comparatively tested against 44 urethral samples. These included the following: 50-test vials, lot 9HZE, from Mallinckrodt, Inc. (MK) (St. Louis, Mo.); 50-test vials, lot 52-58-233, from Associates of Cape Cod, Inc. (CC) (Woods Hole, Mass.); and 50-test vials, lot L14179, from Microbiological Associates (MA) (Walkersville, Md.). The commercially available MK lysate is not extracted by organic solvents, whereas the other two LAL preparations are extracted with chloroform during manufacture. All three LAL preparations had labeled sensitivities of 0.06 ng/ml to the established reference *Escherichia coli* endotoxin (lot EC-2, Bureau of Biologics, U.S. Food and Drug Administration).

For the evaluation of urethritis in 116 men by nonextracted lysate, two additional lots 9IU and 9JZ with minimum sensitivities to the EC-2 standard of 0.04 and 0.05 ng/ml, respectively, were furnished by Mallinckrodt, Inc. Each patient sample was then evaluated by three nonextracted LAL preparations.

LAL assay procedure. Specimens of urethral exudate were thawed and serially diluted in pyrogen-free water to final concentrations ranging from 1:50 to 1: 102,400 of the original sample. The microdilution procedure was used and performed as previously described (6). Briefly, 0.05 ml of the 1:50 dilution was added to wells 1 and 2. Serial twofold dilutions were then made from wells 2 through 12 in water with a 0.05-ml diluter. Each LAL preparation was reconstituted with pyrogen-free water as recommended by the manufacturer, and 0.05 ml of lysate was added to each well. The plates were covered with plastic lids, mixed, and incubated at 37°C for 1 h. The presence or absence of gelation was then determined by adding 0.05 ml of a 0.005% aqueous crystal violet stain solution to each well. The bottoms of the plates were then viewed at an oblique angle (30 to 45°), and gelation was noted in wells in which the stain did not mix and color the contents; lack of gelation was noted in wells in which the stain mixed and colored the contents. The LAL results were read without previous knowledge of the LAL preparation tested or the microbiological findings for each patient.

Statistical analysis. All dilutions were converted to a $\log_2$ value, and the highest dilutions giving positive LAL assays for each LAL preparation were then entered into a Hewlett-Packard model 9825A programmable calculator. The single-tailed Student's paired *t* test was used for statistical analysis.

RESULTS

Comparison of LAL preparations. The percentages of LAL test results that were positive for the three LAL preparations tested comparatively at various dilutions of 28 gonococcal and 16 nongonococcal urethral samples are shown in Table 1. The breakpoint dilutions for differentiation of gonococcal and nongonococcal urethritis for MK, CC, and MA lysates were 1: 1,600, 1:800, and 1:400, respectively, although each LAL preparation had the same sensitivity (0.06 ng/ml) to the endotoxin standard EC-2. The endotoxin activities present at low dilutions in the nongonococcal samples were below detectable levels for each LAL preparation at the appropriate breakpoint dilutions.

The sensitivities to endotoxin in both gonococcal and nongonococcal urethral samples varied among the three LAL preparations with MK being the most sensitive, followed by CC and MA (Table 1). The interlysate statistical comparisons of the sensitivities to the exudate from men with gonococcal and nongonococcal urethritis are shown in Table 2. MK was statistically

more sensitive than both CC and MA ($P < 0.001$) for endotoxin in gonococcal exudate; CC was more sensitive than MA ($P < 0.001$). For nongonococcal samples, MK was not statistically different from CC ($P > 0.05$) but was more sensitive than MA ($P < 0.01$); CC was also more sensitive than MA ($P < 0.01$).

Evaluation of nonextracted LAL preparations. The mean microdilution LAL test results for three lots of nonextracted lysate for urethral samples from men with gonococcal and nongonococcal urethritis serially diluted from 1:50 to 1:102,400 are shown in Table 3. Positive LAL test results were obtained in 100% (61/61) of patients with gonococcal urethritis at dilutions from 1:50 to 1:6,400 of urethral sample. Positive LAL test results were also obtained in a number of patients with nongonococcal urethritis but at lower dilutions (1:50 to 1:800). At a dilution of 1:1,600, however, all but two patients with nongonococcal urethritis were negative for endotoxin activity. The one patient with a positive LAL test to a dilution of 1:51,200 was an established sexual contact with a woman who had a positive culture for *N. gonorrhoeae* and may, therefore, represent a culture failure. There was no statistical difference ($P > 0.05$) between the three nonextracted LAL preparations.

A comparison of LAL test results at a breakpoint dilution of 1:1,600 to Gram-stained smears of 116 patients with culture-proven gonococcal and nongonococcal urethritis is shown in Table 4. The sensitivities and specificities were 95% (58/61) and 98% (54/55) for Gram-stained smears and 100% (61/61) and 96% (53/55) for the LAL test, for cases of gonococcal and nongonococcal urethritis, respectively.

DISCUSSION

In 1964, Levin and Bang (4) first discovered that lysate made from the amoebocytes of the horseshoe crab (*Limulus polyphemus*) forms a gel in the presence of small quantities of bacterial endotoxin. Since then, many procedures have been used to improve the sensitivity and to eliminate the seasonal variations noted with the lysate preparations (3, 11, 13). Sullivan and Watson (J. D. Sullivan and S. W. Watson, U.S. Patent 4,107,077, August, 1978) were granted a U.S. patent for a method to improve the sensitivity of lysate using an organic solvent extraction procedure, and they reported that chloroform extraction produced the best results. Today most commercially prepared lysate is extracted by chloroform to remove inhibitors and improve sensitivity. However, MK lysate (Pyrogent) is not prepared by organic solvent extraction and is available in lots with high sensitivity to standard reference endotoxin.

Comparative results of sensitivity for extracted and nonextracted LAL preparations to gonococcal and nongonococcal urethral samples show that the nonextracted lysate was significantly more sensitive. All three LAL preparations performed satisfactorily in differentiating gonococcal from nongonococcal urethritis since breakpoint dilutions could be determined for each. However, the higher sensitivity displayed by the nonextracted lysate to native endotoxin in gonococcal exudates with its similar sensitivity to nongonococcal endotoxin components would allow a wider range for breakpoint dilutions as shown in Table 3. The need for a more appropriate endotoxin standard is also apparent when testing for native (unpurified) endotoxin in clinical samples since the three LAL preparations tested had the same sensitivities to the purified *E. coli* endotoxin standard EC-2. Variations in sensitivities of different LAL preparations have recently been reported when testing partially purified bacterial endotoxins (12).

The results obtained in the initial evaluation of 116 men with exudative urethritis confirmed our previous findings (7, 9) that use of the LAL assay results in a high degree of sensitivity (>99%) and specificity (>96%) in men. Overall ability to predict culture results was approximately 98% even though the LAL test is not specific for *N. gonorrhoeae*. The endotoxin associated with *N. gonorrhoeae* has been shown to react with the LAL test with as low as 0.07 ng of intact outer membrane per ml (8) and was also shown to be more sensitive than other gram-negative bacteria (10). The LAL test is also

TABLE 3. *Percent positive LAL tests at various dilutions of urethral samples by nonextracted lysate for 116 cases of exudative urethritis in men*

Diagnosis	No. tested	% Positive at dilution 1:											
		50	100	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200	102,400
Gonococcal urethritis	61	100	100	100	100	100	100	100	100	98	95	93	79
Nongonococcal urethritis	55	71	53	20	13	5	4	4	2	2	2	2	0

TABLE 4. *Results of Gram-stained smears and LAL assay with nonextracted lysate for 116 cases of exudative urethritis in men*

Diagnosis	No. of Patients	Gram-stained smears		LAL assay[a]	
		Positive	Negative	Positive	Negative
Gonococcal urethritis	61	58	3	61	0
Nongonococcal urethritis	55	1	54	2	53

[a] Original sample diluted 1:1,600.

sensitive to endotoxin components in the urethral discharge from men with nongonococcal urethritis. The potential causative agents of nongonococcal urethritis have been the focus of recent studies (2), and it is probable that *Chlamydia trachomatis* and *Ureaplasma urealyticum* are the most common urethral pathogens in nongonococcal urethritis (1, 14). Since chlamydiae have been shown to react with LAL (5), it is possible that the false-positive reactions seen at the lower dilutions (≤1:800) may be due to chlamydiae. However, this endotoxin activity was easily removed by proper dilution, while a high degree of sensitivity to *N. gonorrhoeae* was retained. Similar results using the LAL test in the initial evaluation of gonococcal and nongonococcal cervicitis in women have recently been reported (10).

The nonextracted lysate performed very well in the rapid differentiation of gonococcal and nongonococcal urethritis in men. The three different lots, which varied in sensitivity from 0.04 to 0.06 ng/ml (EC-2), each differentiated both diseases at the breakpoint dilution of 1:1,600. A wide dilution range of clinical samples was also apparent, thus minimizing the possible variations in test results due to small variations in the amount of specimen collected with the syringe.

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