JOURNAL OF CLINICAL MICROBIOLOGY, June 1985, p. 865–868
0095-1137/85/060865-04$02.00/0
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Vol. 21, No. 6

Detection of Endotoxin in the Plasma of Patients with Gram-Negative Bacterial Sepsis by the *Limulus* Amoebocyte Lysate Assay

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Received 26 November 1984/Accepted 25 February 1985

A total of 120 *Limulus* amoebocyte lysate (LAL) determinations were made on plasma obtained from normal, healthy human blood donors. Results demonstrated a mean endotoxin level in blood of 0.02 to 1.57 pg/ml. The amount of *Escherichia coli* endotoxin added to human plasma samples can be quantitated by both nephelometry and turbidimetry. Endotoxin-spiked samples were shown to be significantly different from unspiked samples. When plasma samples were collected from 45 patients hospitalized at three centers, a strong association was demonstrated between a positive *Limulus* amoebocyte lysate assay and a septic condition. Sensitivity, specificity, and false-positive and false-negative rates for the *Limulus* amoebocyte lysate assay as a diagnostic test for gram-negative bacteremia were estimated.

The *Limulus* test described by Levin and Bang is the most sensitive method of detecting gram-negative bacterial endotoxin (J. Levin, Fed. Proc. 27:355, 1968). The *Limulus* amoebocyte lysate (LAL) assay has been widely used for in-process and final-release pyrogen testing of medical devices and large- and small-volume parenterals (12, 15). Although the potential clinical utility of this assay has been encouraging for the rapid diagnosis of gram-negative bacterial meningitis (17) and urinary tract infections (9), investigations remain divided on its usefulness in diagnosing gram-negative bacterial sepsis.

A number of LAL test inhibitors have been demonstrated in human plasma (10, 16, 21). In an attempt to circumvent LAL test inhibition, various plasma treatments have been used, including chloroform extraction (11), pH adjustment (5), platelet extraction (3), heat and dilution (2), gel filtration (8), ammonium sulfate precipitation (6), ether extraction (7), and addition of perchloric acid (13).

The present study was undertaken to determine the average amount of circulating endotoxin in platelet-rich plasma (PRP) obtained from a population of healthy humans. It was also designed to ascertain whether endotoxin levels rise and fall at different times of the day, whether they are affected by fasting or varied meal intake, and whether *Escherichia coli* endotoxin added to samples of normal human plasma can be recovered and quantified. Two quantitative methods, nephelometry and turbidimetry, were used to measure endotoxin levels.

In another part of the study, PRP collected from hospitalized patients was subjected to the quantitative nephelometric procedure and to a newly introduced chromogenic procedure.

MATERIALS AND METHODS

Test and reference substances. (i) Healthy donors. Blood samples were taken twice on the same day from 30 healthy male and female human volunteers. One sample was taken in the morning, after an overnight fast; another was taken approximately 2 h after lunch. Morning and afternoon samples were coded to distinguish between them, and each sample was divided into two batches. One batch was used to determine background endotoxin levels and to set up a standard endotoxin curve. The other batch was spiked with O55:B5 *E. coli* endotoxin (Difco Laboratories) to a level of 1,000 pg/ml.

(ii) Hospitalized donors. Plasma samples were obtained from 45 patients hospitalized at three centers. These patients had either symptoms of recognized septic shock or fevers of unknown etiology. In each case, samples were taken for simultaneous blood culture and LAL evaluation. LAL assay results were recorded before the results of blood cultures were known.

Sample collection and processing. Sterile, empty, nonpyrogenic 10-ml evacuated blood collection tubes were used. Nonpyrogenic sodium heparin (150 U) was injected through a tuberculin syringe into the Vacutainer (taking care not to break the vacuum). The tubes were then used to collect blood samples by the standard aseptic technique. To prepare PRP, blood samples were centrifuged at 150 × *g* for 10 min. The separated plasma fraction was apyrogenically removed with a depyrogenated pipette and transferred to depyrogenated test tubes (10 by 75 mm). After the plasma fractions were collected, it was necessary to remove the inherent LAL inhibitors found in plasma. This was done by transferring a 0.2-ml sample of the plasma fraction to a pyrogen-free test tube (10 by 75 mm) and diluting it with 1.8 ml of pyrogen-free water for injection (1:10 dilution). The mixture was vortexed for about 10 s, sealed with double Parafilm, and heated for 10 min at 70 ± 2.0°C. Vortexing was then repeated for another 10 s.

LAL test systems. (i) Nephelometric. A 0.05-ml portion of the heat-treated plasma supernatant was placed in a pyrogen-free test tube, and 0.1 ml of reconstituted lysate (lot 76-6, containing 25 mM $MgCl_2$; Travenol Laboratories) was added. Blanks of each specimen were prepared by combining 0.05 ml of heat-treated plasma with 0.1 ml of pyrogen-free water for injection. All the tubes were then covered with Parafilm, mixed gently, and incubated at 37°C for 60 min. After incubation, 0.2 ml of sodium dodecyl sulfate (3% [wt/vol]) was added to the tubes; the tubes were then agitated gently and left for 3 to 5 min. A 1-ml portion of carboxymethyl cellulose (0.08% in saline) was then added, and the tubes were read in a Hyland nephelometer at its fixed wavelength and in a spectrophotometer at 360 nm. Standard control curves of 200, 100, 50, 25, and 0 pg/ml were prepared with *E. coli* O55:B5 endotoxin, which was added to

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TABLE 1. Quantitative analysis of normal human plasma for endotoxin equivalent concentration

Sample no.	Endotoxin concn (pg/ml) from subjects who had:			
	Fasted		Not fasted	
	Nephe-lometer	Spectropho-tometer	Nephe-lometer	Spectropho-tometer
1	0.000	0.000	1.715	0.000
2	0.741	0.000	1.596	0.000
3	0.000	0.000	16.621	0.000
4	0.000	0.000	0.000	0.000
5	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000
7	0.000	0.000	0.000	0.000
8	0.000	0.000	0.000	0.000
9	0.000	0.000	0.000	0.000
10	0.000	0.000	0.202	0.000
11	0.000	0.000	0.000	0.000
12	0.000	0.000	0.000	0.000
13	0.000	0.000	0.000	0.000
14	0.000	0.000	0.000	0.000
15	0.000	0.000	0.000	0.000
16	0.000	0.000	0.000	0.000
17	0.000	0.000	0.000	0.000
18	0.000	4.291	5.519	0.728
19	0.000	0.000	0.000	0.000
20	0.000	0.000	0.000	0.000
21	0.000	0.000	0.000	0.000
22	0.000	0.000	11.376	0.000
23	0.000	0.000	0.000	0.000
24	0.000	0.000	0.000	0.000
25	0.000	0.000	10.145	1.077
26	0.000	0.471	0.000	0.000
27	0.000	4.365	0.000	0.000
28	0.000	0.000	0.000	0.000
29	0.000	0.000	0.000	0.000
30	0.000	0.000	0.000	0.000

negative human plasma. These controls were treated exactly the same as the test articles.

(ii) Chromogenic. A daily working endotoxin standard was prepared in a depyrogenated test tube by adding 0.1 ml of a 50-ng/ml endotoxin solution to 10 ml of the Tween 20 (positive control diluent). This standard was prepared on the day it was to be used.

Four endotoxin control concentrations were prepared in depyrogenated test tubes by diluting 1.0 ml of the 0.5-ng/ml endotoxin solution with 4.0 ml of positive control diluent (100 pg/ml). A 1.0-ml portion of the 100-pg/ml solution was used to prepare three serial twofold dilutions with the positive control diluent (50, 25, and 12.5 pg/ml). The positive control diluent was also used as a negative control.

Samples (0.05 ml) of the negative control solution, the test sample, and each of the endotoxin control solutions were poured into three test tubes each. An additional 0.05 ml of positive control diluent was placed in one test tube to serve as the diluent blank. A 0.05-ml sample of each PRP tested served as the PRP blank (optical density reference blank).

The LAL vial was reconstituted with 3.0 ml of cold lysate diluent. The lysate (0.1 ml) was added to the opposite wall of tubes containing the negative controls, test samples, or endotoxin control standards, in that order. Reconstituted LAL diluent (0.1 ml) was added to all blanks. The test tubes were then covered with a sheet of Parafilm and agitated slightly to mix the samples, after which they were incubated in a 37 ± 0.5°C water bath or heating block for 10 min. On removal, the test tubes were placed in an ice bath.

Cold chromogenic substrate (0.5 ml) was added to all tubes. Tubes were agitated and placed in a 37 ± 0.5°C water bath for 15 min. On removal, test tubes were placed in an ice bath. After addition of 0.1 ml of 5.0% acetic acid, tubes were again agitated.

Optical density was read at 405 nm on a spectrophotometer. The spectrophotometer was set to zero with fresh distilled water, after which the diluent blank, the PRP blank, the negative control samples, the test samples, and the endotoxin control standards were read and recorded in that order. The optical density value of the diluent blank was subtracted from that of the endotoxin control standards. The optical density value of the donor (PRP) sample blank was subtracted from that of the corresponding donor test sample. The test sample optical density figure was compared with that of the endotoxin standard curve to determine the picogram equivalent levels of the test sample.

For both the nephelometric and chromogenic LAL assays, test samples (from hospitalized patients) with endotoxin concentrations that exceeded the highest endotoxin standard were subsequently rediluted with pyrogen-free water (1:20, 1:40, or accordingly) and retested. All test articles were evaluated in a blind fashion.

Statistical methods. To compare the endotoxin concentration levels for samples from fasted and non-fasted subjects, a sign test (1) was applied. Volunteers for whom the results both after fasting and not fasting were 0.000 pg/ml (i.e., ties) were ignored in performing the sign test.

For the plasma samples from the 45 hospitalized patients, results from the blood cultures and the LAL tests were compared. LAL tests were performed via the nephelometric assay for patients 1 to 7 and via the chromogenic assay for patients 8 to 17. Results from both LAL methods were combined in estimating test sensitivity, specificity, and false-positive and false-negative rates. There were not sufficient data to indicate any differences between the two LAL methods with respect to these four parameters. Individual two-sided 95% confidence intervals were estimated for each of the four parameters.

RESULTS

Table 1 gives the endotoxin content, measured by nephelometric and turbidimetric techniques, of plasma samples taken from 30 normal human subjects after overnight fasting and after lunch. Quantitation was performed by comparison with control curves obtained by measuring negative human plasma spiked with *E. coli* O55:B5 endotoxin at 0, 25, 50, 100, and 200 pg/ml. The turbidimetric analysis did not detect any significant differences between the endotoxin equiva-

TABLE 2. Mean estimated endotoxin concentrations in human plasma

Sample	Endotoxin concn (pg/ml) by:			
	Nephelometer[a]		Spectrophotometer[b]	
	F[c]	NF[c]	F	NF
Spiked[d,e]	1160.3	1196.6	1274.1	1456.5
Unspiked[d]	0.0247	1.57	0.30	0.06

[a] Correlation coefficient (r) = 0.96.
[b] Correlation coefficient (r) = 0.95.
[c] F, Samples taken from fasted subjects; NF, samples taken from non-fasted subjects.
[d] Mean of 30 samples.
[e] Results have been multiplied by 10 to compensate for the 1:10 dilution of the plasma.

lents in samples taken from the fasted and non-fasted subjects.

For the nephelometric procedure, samples taken from 7 of the 30 non-fasted patients gave nonzero results. For all seven, the result obtained from non-fasted patients exceeded that obtained from fasted patients. Although this difference is statistically significant ($P < 0.01$ for a sign test), the magnitude of the differences has little practical significance.

The samples that were taken from the same donors and spiked with *E. coli* endotoxin at a level of 1,000 pg/ml were analyzed in a blind fashion by both instrumental techniques. The results of this study are given in Table 2, along with mean estimates of the endotoxin equivalents of the unspiked samples.

Results of analyses of individual samples from hospitalized patients are shown in Table 3. A total of 17 LAL determinations were made on patients who had samples that were either LAL positive or culture positive (7 patients tested by the nephelometric LAL assay and 10 patients tested by the chromogenic LAL assay). Of the 17 patients, 5 had gram-positive culture-proven bacteremia, and their samples exhibited a negative LAL result. Since there are reports of LAL-positive results in the presence of gram-positive culture-proven bacteremia, the latter finding is encouraging with regard to test specificity (18, 19). Of samples taken from 10 patients having culture-proven gram-negative bacteremia, one sample was negative by LAL. Two individuals had samples that were LAL positive and culture negative. An additional 27 PRP samples collected from hospitalized patients were found to be blood culture negative and negative by the LAL assay. Overall results for the hospitalized patients are summarized in Table 4. There is a strong association between the culture results and the LAL test results ($P < 0.001$ by Fisher's exact randomization test). Estimates of test sensitivity, specificity, and false-positive and false-negative rates for use of the LAL test as a diagnostic test are also given in Table 4.

TABLE 3. Comparison of LAL-positive patient plasma and culture-proven gram-negative bacteremia

Assay method and patient no.	Culture result	Endotoxin concn (pg/ml)
LAL nephelometric determination		
1	*E. coli*	830
2	*Klebsiella*	270
3	*Staphylococcus aureus*	0
4	Enterococcus	0
5	*E. coli*	280
6	*E. coli*	450
7	*E. coli*	118
LAL chromogenic determination		
8	GNB[a]	850
9	GNB	850
10	GNB	120
11	Culture negative	230
12	*S. aureus*	0
13	*E. coli*	650
14	Culture negative	230
15	Enterococcus	0
16	*Staphylococcus*	0
17	*Klebsiella*	0

[a] GNB, Gram-negative bacteremia.

TABLE 4. Summary of LAL results for diagnosis of gram-negative bacteremia

Culture results	LAL results[a]	
	Negative	Positive[b]
Negative	27	2
Gram-positive bacteremia	5	0
Gram-negative bacteremia	1	9

[a] Sensitivity is estimated at 0.900 with a 95% confidence interval of 0.555 to 0.997; specificity is estimated at 0.941 with a 95% confidence interval of 0.803 to 0.993; false-positives are estimated at 0.182 with a 95% confidence interval of 0.023 to 0.518; and false-negatives are estimated at 0.030 with a 95% confidence interval of 0.001 to 0.158.

[b] LAL positive is defined as an endotoxin concentration exceeding 100 pg/ml.

DISCUSSION

The results of this study demonstrate that plasma taken from healthy donors does not contain significant amounts of endotoxin when assayed by the test systems reported here. *E. coli* O55:B5 added to the plåsma of the same individuals could be recovered when the plasma was diluted 1:10 and treated at 70°C for 10 min. By using the same plasma sample preparation technique, we could detect endotoxin in the majority of patients also having culture-proven gram-negative bacteremia. Both the nephelometric and chromogenic LAL methodologies appear to be equally effective. These results tend to confirm and extend the studies of Cooperstock et al. (2) and Das et al. (3). Five plasma samples from patients with culture-proven gram-positive bacteremia were LAL negative. These results, which suggest LAL test specificity for endotoxin, do not agree with the results of other studies (4, 19). However, it should be kept in mind that LAL test results are specific to the test methodology used and probably are also specific to the LAL reagent used as well. There are major manufacturing differences among commercial LAL reagents. These manufacturing differences clearly lead to differences in test results when various commercial lysates are compared by using the same test substance (14, 18, 20). This observation could also hold true for the interaction of plasma with various commercial LAL assays. This is the first time that data have been published on the Travenol LAL reagent for the detection of endotoxin in plasma. This reagent is not commercially available.

The results of these initial studies are encouraging with regard to LAL test specificity and sensitivity. The findings suggest that the clinical utility of the LAL test for the detection of gram-negative sepsis should be reevaluated in light of new information, modified reagents, and revised test systems.

ACKNOWLEDGMENTS

We gratefully acknowledge the statistical input of H. Sargent, Travenol Laboratories, Inc.

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