A Technique for Quantitative Measurement of Endotoxin in Human Plasma[1] (35572)

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The quantitative measurement of endotoxin in plasma is a prerequisite for defining the role of endotoxin in clinical gram-negative sepsis. Circulating endotoxin has been identified in 30 of 81 patients with gram-negative sepsis by modifications of the skin sensitivity test of Thomas (6, 13, 14). This test however has serious limitations because it will not reliably detect less than 1 μg/ml of plasma, and because the time, equipment, and personnel it requires make large-scale clinical trials prohibitive.

The *Limulus* coagulation test, as first described by Levin and Bang (8) and subsequently by Rojas-Corona *et al.* (15), shows greater promise as a useful clinical method for the quantitative measurement of endotoxin. Its advantages are simplicity and speed, for it is an *in vitro* test using stable reagents giving results in 90 min. This report deals with modifications of the *Limulus* technique which make it possible to obtain reliable quantitative determinations of endotoxin in human and rabbit plasma. Additional data are given showing that the test is specific for endotoxin and does not yield false positives with other bacterial toxins.

Methods. 1. Preparation of Limulus lysate. All equipment is rendered free of endotoxin by washing with distilled water, steam sterilization, and dry heat at 170° for 2 hr. As per the method of Levin and Bang (10), a 13-gauge pyrogen-free needle is inserted into the ventral sinus of the cardiac chamber at the junction of the cephalothorax of the horseshoe crab (*Limulus polyphemus*), and the blood (200 ml/crab, 10 in. across the carapace) is drained into siliconized flasks containing an equal volume of 0.125% *N*-ethylmaleimide in sterile pyrogen-free 3% sodium chloride, pH = 7.4, with Tris buffer. The cells (amebocytes) are harvested by either gravity sedimentation for 90 min in a 1-liter Ehrlenmeyer flask or centrifugation at 600 rpm for 10 min. All glassware for harvesting the lysate must be siliconized to prevent adherence of the amebocytes to the walls of the flasks. *N*-Ethylmaleimide acts as an anticoagulant and stabilizes the cell membrane during the washing process. However, exposure to *N*-ethylmaleimide for more than 120 min prevents adequate lysis with distilled water and therefore limits the sedimentation time. Centrifugation must be gentle to prevent premature cell disruption and coagulation of the *Limulus* blood.

Each 5 ml of packed amebocytes is resuspended and washed three times with 30 ml of sterile pyrogen-free 3% sodium chloride. After the final wash, the cells are ruptured by the addition of 30 ml of distilled water/5 ml of packed amebocytes. After thorough mixing, the cell suspension is allowed to stand for a minimum of 6 hr and preferably 12 to 24 hr at 4°. The cell debris is removed by centrifugation and the clear supernatant is ready for use. This lysate is stable at 4° for many months and indefinitely if frozen. On standing at 4°, the lysate frequently develops a small amount of flocculation which can be removed by centrifugation. The resultant clear supernatant retains its full activity and should be used to simplify reading the reaction for viscosity and gel formation.

Stability of the lysate during storage at 4°

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should be checked periodically with plasma containing a known concentration of endotoxin. Potent lysate will consistently give a positive test with a stock solution of the endotoxin (*Serratia marcescens* LD_{50} mouse = 610 μg, A. Nowotny, Temple University, Philadelphia) containing at least 0.002 μg/ml (see Fig. 3).

2. Preparation of plasma. A 0.1-ml sample of 25% glacial acetic acid (2.9 *N*, pH 2.2) is added to 1.0 ml of heparinized plasma to lower the pH to 4.0 ± 0.1. Plasma pH is returned to 6.2 ± 0.1, by the subsequent addition of 0.2 ml of 50% (w/v) anhydrous dibasic potassium phosphate buffer (50% K_2HPO_4, pH 9.4). Periodic pH determination of the treated plasma is necessary to confirm the stability of the buffers, for this reaction is pH dependent.

Lowering the pH of plasma to 6.2 without the intermediary step is unsatisfactory because flocculation and coagulation of heparinized plasma will result from the precipitation of fibrinogen and euglobulin.

Heparinized plasma should be used to detect circulating endotoxin for several reasons. Since no significant amount of endotoxin resides in the cellular compartment of whole blood, the cells act as a diluent and so decrease the sensitivity of the technique (3). Moreover, if they sludge on incubation, they interfere with reading the end point. Calcium depleting anticoagulants are to be avoided for Skarnes and Chedid (19) have reported that reduced ionic calcium activates serum esterase capable of detoxifying endotoxin *in vitro*. EDTA blocks the *Limulus* reaction (8). The possibility that coagulation may trap some of the macromolecular endotoxin makes plasma preferable to serum (11).

Endotoxin added to normal plasma in concentrations as high as 500 μg/ml are undetectable by the lysate technique unless the plasma is diluted with normal saline. Figure 1 demonstrates that, with dilution of plasma, the entire amount of endotoxin is recoverable. This evidence as well as the recent work of Levin *et al.* (11) suggests endotoxin binding by plasma proteins. Shifting the pH of rabbit and human plasma causes precipitation of euglobulin and permits detection of endotoxin by the lysate, thus confirming the work of Rudbach that lipopolysaccharide binds to the globulin fraction and not to fibrinogen or albumin (17).

3. Limulus technique. 0.1 ml of *Limulus* lysate is reacted with 0.1 ml of treated plasma (pH 6.2 ± 0.1) for 60 min at 37° in sterile, sealed pyrogen-free 10 × 75-mm disposable test tubes. While the reaction will proceed to gelation over a wide temperature range of 20–40°, 37° is optimal for speed and

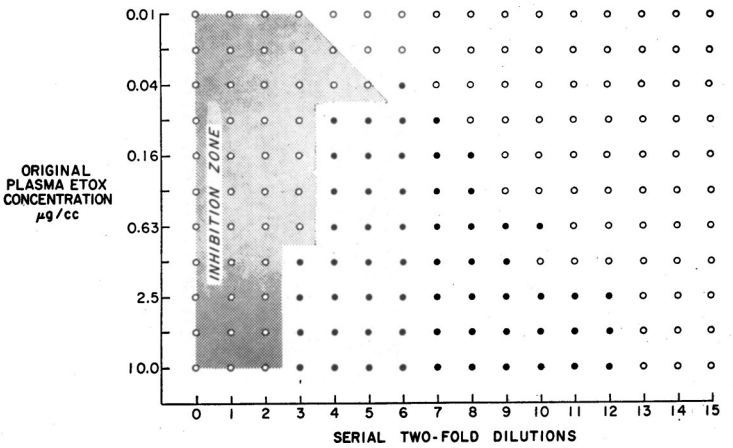

FIG. 1. Endotoxin detection in untreated human plasma: Endotoxin (*S. marcescens*, Nowotny) was added to 1 ml of a solution containing saline (0.1 ml) and plasma (0.9 ml) in different amounts as shown on the ordinate. A series of twofold dilutions in saline of 0.1 ml from each of the 11 different concentrations was prepared as shown on the abscissa. (●) Positive tests; (○) negative tests.

reproducibility. The presence of increased viscosity or solid gel formation when the tube is inverted after incubation represents a positive test for endotoxin. Gel formation in most instances is prevented by agitation of the tubes prior to the end of the incubation period.

Serial twofold dilutions permit definition of the endotoxin titer by the formula:

$$[ETOX]_0 = [ETOX]_{ep}/\text{Dilution},$$

where $[ETOX]_0$ = endotoxin concentration of the original sample and $[ETOX]_{ep}$ = endotoxin concentration at the end point as determined by the known plasma standards. Dilutional error is ± 1 dilution. Accuracy increases where the level detected approaches the concentration of the end point. This system is capable of detecting 0.005 to 0.001 μg of purified endotoxin/ml of treated plasma. Endotoxin can be measured quantitatively in both rabbit and human plasma by this technique.

Varying proportions of lysate and plasma were examined to determine the optimal mixture for maximum sensitivity. Sensitivity is not improved by as much as a fourfold increase in either plasma or lysate, or by increasing the volume of both plasma and lysate from 0.1 up to 1.0 ml. For convenience, and to conserve the lysate, the volume of each is kept at 0.1 ml. The pH studies have demonstrated the optimal range to be from 5.5 to 7.5; below 5.3 the reaction will not proceed to gelation. The reaction is considered positive only if it proceeds to solid or near solid gelation. Flocculation is considered negative. Levin has demonstrated a relationship between the endotoxin titer and the time of onset of increasing viscosity or gelation (10), but electronic measurement of this relationship has not yielded uniformly reliable results in our hands.

Results. Rojas-Corona *et al.* (15) demonstrated that normal plasma or its vasoactive constituents do not produce gelation of the lysate. We have confirmed this observation, and extended it to include calcium, hemoglobin, and thrombin. To demonstrate the specificity of the *Limulus* lysate for endotoxin, as distinguished from other bacterial substances, a double blind study was conducted in conjunction with Drs. Kelsey Milner and Jon Rudbach (National Institutes of Allergy and Infectious Diseases, Hamilton, Montana). Table I shows the result of a double blind comparative assay of 15 separate samples tested by the *Limulus* technique, the intravenous chick embryo assay, and the fever index of rabbits. Correlation between the three tests was good at both extremes of endotoxin activity, with one exception. *Sal-*

TABLE I. Double Blind Comparative Assay for Endotoxin.[a]

No.	Specimen	Gelation of lysate[b] (μg)	ivCELD$_{50}$[c] (μg)	FI$_{40}$[d] (μg)
1	Ec 79/83 ET	0.003	0.0045	1.1
2	Ec 178 ET	0.007	0.0025	—
3	Ec 93/94 ET	0.007	0.0016	0.41
4	Ec 177 ET	0.013	0.0021	1.1
5	Se 388 ET	0.033	16	0.51
6	Ec 0127 TE (DIFCO)	0.20	0.19	19.0
7	Ec 23-27 ET	0.26	—	0.17
8	Ec 148/149 ET	0.67	0.11	—
9	Ec 79/83 NH	26	—	1000+
10	Se 326-29 AH	125	—	1000+
11	Ec 72/76 NH	200	34.0	1000+
12	Se 326-29 NH	200	—	1000+
13	Ec 178 NH	250	—	1000+
14	Ec 58/60 Residue	500	3.6	520+
15	Ec 93/94 NH	500	24	1000+

[a] An unknown test substance was considered positive for endotoxin if gelation of the lysate or an ivCELD$_{50}$ was obtained with less than 1 μg, or if FI$_{40}$ was obtained with less than 10 μg. The test substances are classified by their known chemical characteristics and are listed in the order of their decreasing reactivity with the lysate. Because of its unusual CELD$_{50}$, Se 388 ET was assayed in triplicate with identical results. Abbrev.: Ec = *E. coli*; Se = *S. enteriditis*; ET = endotoxin (aqueous phenol extraction); NH = native hapten [trichloroacetic acid extraction of bacterial protoplasm per method of Anacker *et al.* (1)]; AH = acid hapten [acetic acid hydrolysis of endotoxin for 180 min at 100° as per method of Freeman (7)].

[b] Minimum micrograms to produce lysate gelation, performed by Reinhold.

[c] Minimum micrograms to produce intravenous chick embryo LD$_{50}$, performed by Milner.

[d] Minimum micrograms to produce fever index$_{40}$ in rabbits, performed by Milner.

monella enteriditis 388 endotoxin was active by the lysate technique and rabbit fever index, but inactive by the chick embryo assay. Since there is such close agreement between the results of the lysate technique, the fever index, and the chemistry, it is reasonable to assume the chick embryo assay was in error. The variation in biologic activity of the eight endotoxins is to be expected, for there are grades of purity depending upon the care and sophistication of the techniques used in preparation. The lysate test did not yield a single false positive or false negative reaction for endotoxin. All endotoxins tested to date including other preparations from *S. marcescens* and *Salmonella typhi* have yielded positive results.

Additional evidence that the *Limulus* lysate test is specific for endotoxin was obtained by the observation that the lysate does not gel when interacted with aliquots of pure log phase cultures (10^9 viable organism/ml) of pathogenic gram-positive bacteria (pneumococcus, *Staphylococcus aureus* or *Streptococcus hemolyticus*), with activated streptolysin (Behringwerke AC), streptodornase (1000 units/ml) or streptokinase (4000 units/ml), or with concentrated clostridial tetanus toxin (50–70 μg/ml).

Figure 2 demonstrates the accuracy of the test for the quantitative measurement of endotoxin in samples of normal heparinized plasma to which known quantities of endotoxin have been added. Stock solutions of the endotoxin (*S. marcescens* LD_{50} mouse = 610 μg) were added to whole plasma in a ratio of 1:9 to produce original concentrations in the range of 1.0 to 0.001 μg/ml of plasma. After treatment with 25% acetic acid and 50% K_2HPO_4, serial twofold dilutions of each concentration were performed 10 times and each of the original samples and its dilutions were tested by the *Limulus* technique. The resulting pattern of positive and negative tests demonstrates the reliability of the technique at concentrations above and below the end point. This distribution is identical for the endotoxin standards in saline and rabbit plasma and documents the validity of the formula above.

Figure 3 demonstrates the consistency of this test at concentrations of 0.002 μg/ml of plasma or greater. Such concentrations will yield a 90+% incidence of positive tests, a degree of reliability unequaled by any other assay for endotoxin. Similarly the test is 100% negative at concentrations below 0.0005 μg/ml. All control samples of human (n = 30) and rabbit plasma (n = 65) have been negative to date. The negative controls and the quantitative relationship between the plasma standards and their dilutions rule out the possibility that other normal plasma constituents produce gelation of the lysate.

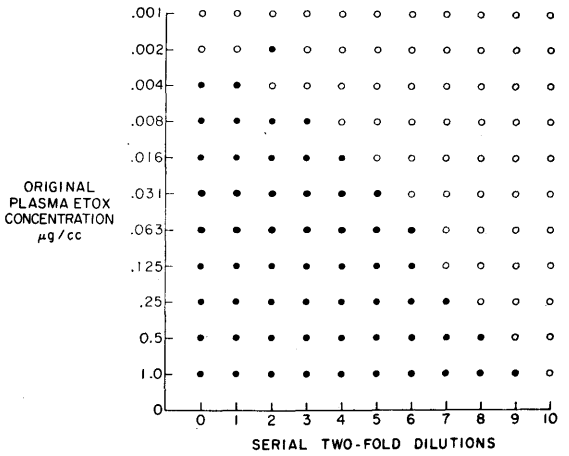

FIG. 2. Endotoxin detection in treated human plasma: Plasma endotoxin standards (*S. marcescens*, Nowotny) were prepared in a manner identical to Fig. 1 except for treatment with 25% glacial acetic acid and 50% K_2HPO_4 prior to dilution and testing with lysate. (●) positive tests; (○) negative tests. $[ETOX]_{ep} = ETOX_0 \times$ dilution = 0.002–0.001 μg endotoxin/ml (see text).

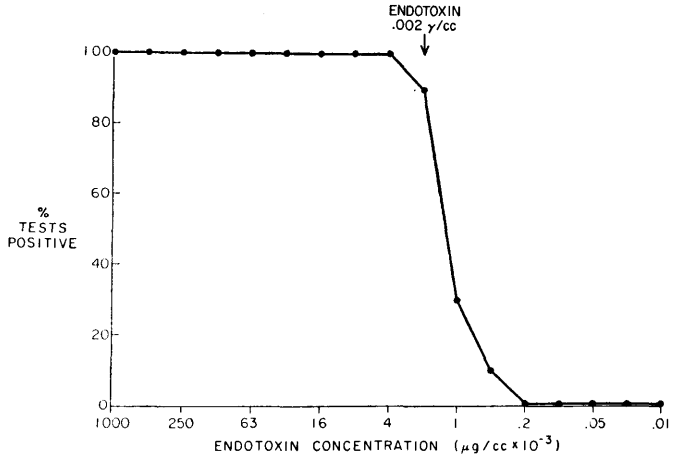

FIG. 3. Lysate detection of duplicate plasma exdotoxin standards ($n = 10$): Duplicate samples ($n = 10$) of plasma endotoxin standards. (*S. marcescens*, Nowotny) were tested in titers from 1.0 to 0.00001 μg endotoxin/ml of plasma.

The usefulness of this technique for monitoring *in vivo* clearance of intravenously injected endotoxin (*S. typhi* 0901, aqueous phenol extraction, ivCELD$_{50}$ = 0.015 μg, J. Rudbach) is indicated by the clearance curves for three series of male New Zealand rabbits ($n = 5$) receiving 10, 100, and 1000 μg/kg, respectively, after light Nembutal anesthesia (Fig. 4). In the group receiving 10 μg/kg, there is no measurable endotoxin circulating within a few minutes, thus confirming previous observations with ^{51}Cr labeled endotoxin (4, 5). In the group receiving 100 and 1000 μg/kg, approximately 75 to 90% disappeared within 15 min, followed by a slower clearance rate with a mean half life of 71 and 132 min respectively. These data show that with the *Limulus* lysate technique it is possible to determine the rate of clearance of varying doses of intravenous endotoxin, that the clearance is biphasic except for a very small dose, and that the rate of the second phase (T 2) varies inversely with the dose.

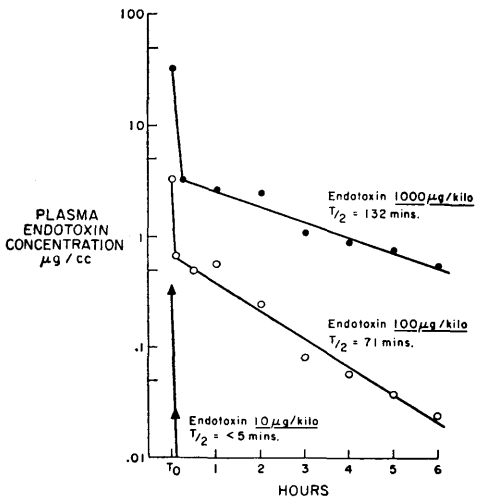

FIG. 4. Clearance of iv endotoxin: Initial endotoxin titer calculated by dividing dose/kilo by measured plasma volume. Subsequent titers determined by formula as defined in text. Each point is mean value for five rabbits. SE$_{10\ \mu g/kg}$ = ±6 min; SE$_{100\ \mu g/kg}$ = ±11 min; SE$_{1000\ \mu g/kg}$ = ±19 min.

Discussion. Limulus polyphemus is one of the oldest phylogenetically intact marine invertebrates known. Fossile specimens as old as 300 million years have been discovered. The observation by Bang (2) and Shirodkar *et al.* (18), of the similarity between diffuse intravascular coagulation in the horseshoe crab and in the experimental mammal during gram-negative sepsis, led to investigations into the behavior of *Limulus* blood on exposure to endotoxin. *In vitro* exposure to endotoxin results in amebocyte aggregation and degranulation. The active principle is contained within the amebocyte, probably the refractile granules, and is released from the cell by lysis with distilled water (9). Electrophoresis and electrofocusing studies of the lysate has revealed the presence of at least

six separate protein moieties. Gelation results in the consumption of all six lysate proteins and renders the gel supernatant protein free. Further work on isolation of the active protein fraction of *Limulus* lysate is in progress.

With the exception of the *Limulus* technique, all tests for endotoxin with a sensitivity of 1 μg or less are bioassays and incapable of yielding rapid quantitative results. Although the best of the reported bioassays do detect as little as 0.001 to 0.005 μg (3), they are much slower and more complicated to carry out.

The problem of plasma protein inhibition and/or detoxification of endotoxin has plagued many investigators. Most bioassays, including the modified skin epinephrine test of McGill *et al.* (13), are sensitive enough to detect endotoxin in saline, but few, previous to this report, have actually measured endotoxin added *in vitro* to plasma. Indeed, Rudbach *et al.* (16) have reported failure to detect up to 100 μg/ml of plasma by the fever index in rabbits. One should therefore hesitate to accept the reliability of any assay unless it can measure endotoxin in plasma standards *in vitro*.

Levin *et al.* (11) have reported the use of chloroform to eliminate the plasma protein inhibitors of the reaction between endotoxin and the lysate. In our hands, this technique is more cumbersome and more time consuming than the pH shift method described here. Moreover, it does not yield reliable quantitative results in serial dilutions. It is important to note that both methods deal with detection of endotoxin in rabbit and human plasma. The exact nature of inhibitors in human plasma, as well as other species, is currently under study.

To date, circulating endotoxin, in titers of 0.002–0.50 μg/ml plasma, has been demonstrated in this laboratory in 18 patients, 12 with gram-negative sepsis. Levin *et al.* have recently reported circulating endotoxin in 17 of 98 patients (12) with the chloroform modification of the lysate technique, using chloroform for removal of inhibitors.

Conclusion. The modifications of the *Limulus* lysate techniques described in this report make this test useful for *in vitro* quantitation of endotoxin in plasma as well as in saline.

1. The specificity of the test for endotoxin as distinguished from other bacterial toxins and other bacterial substances has been demonstrated.

2. Accuracy of the technique can be confirmed by serial dilutions of known standards of endotoxin in plasma.

3. The end point is discrete and easily recognizable at levels of 0.005 to 0.001 μg/ml of plasma, a sensitivity comparable to the most sensitive of all well-documented bioassays.

4. The speed and simplicity of the technique as modified, together with its specificity and sensitivity, give it considerably greater clinical and research potential for the quantitative measurement of circulating endotoxin than other available methods.

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