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Measurement of Bound and Free Endotoxin by the Limulus Assay (38240)

JAMES H. JORGENSEN[1] AND RODNEY F. SMITH[2]
(Introduced by J. A. Bass)

Division of Microbiology, Shriners Burns Institute and the Department of Microbiology, The University of Texas Medical Branch, Galveston, Texas 77550

Early investigators of endotoxin employed filtrates of old cultures of gram negative bacilli as their toxin source on the assumption that the active material was released only upon autolysis of the cells. However, as early as 1917, Ecker demonstrated that material with the properties of endotoxin was released into the culture fluids of young, actively growing cultures of gram-negative bacteria in the absence of any signs of autolysis (1).

The term "free endotoxin" was probably first used by Crutchley, *et al.* in 1967 to describe the lipopolysaccharide (LPS) released into culture fluids by *Escherichia coli* (2). They postulated that free endotoxin was due to a metabolic overproduction of cell wall material during vigorous growth in an aerated liquid medium. However, it has also been shown that simple heat treatment of cells in sodium chloride can cause release of up to one-half the total LPS content of cells (3). Furthermore, Rogers in 1971, demonstrated that the release of LPS from *E. coli* could be effected by a warm water treatment with 0.1 *M* Tris without loss of viability of the cells (4). These findings strongly suggest that LPS may be a readily solubilizable component of the cell wall.

Since endotoxin is a complex molecule composed of both lipid and polysaccharide, identification of the specific component responsible for toxicity has been attempted. Acid hydrolysis of LPS yields lipid A and polysaccharide hapten. However, fractions derived from this harsh treatment have generally been found to contain only about 1% of the biological activity of the parent compound (5).

Another approach to defining the toxic moiety has been to utilize mutants in which the biosynthesis of the majority of the polysaccharide portion of the LPS has been blocked. Such mutants contain only the R-region which is composed of primarily lipid A and core oligosaccharide. Lipopolysaccharide preparations from R-strains have been shown to possess considerable biological activity. These findings have been interpreted to mean that lipid A is the major toxic constituent (6). However, other authors continue to argue that the toxicity of endotoxin is not attributable to a single component of the LPS, but rather a toxic conformation assumed by the entire complex (7).

An assay based on the gelation of amebocyte lysate from *Limulus polyphemus,* the horseshoe crab, has been described as the most sensitive method presently available for the detection of endotoxin (8). This technique has been used successfully for monitoring endotoxemia during shock in experimental animals (9), for detection of gram negative bacteriuria in patients (10), and as a method of detecting endotoxin in parenteral pharmaceuticals (11). The Limulus test has also been evaluated by several investigators as a diagnostic test for patients with gram-negative septicemia or endo-

[1] Present address: Department of Pathology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284.

[2] Present address: Microbiology Laboratory, Alta Bates Hospital, Webster and Colby Streets, Berkeley, California 94705.

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toxemia (12, 13). These latter studies seem to indicate that certain patients have circulating endotoxin in their blood without the presence of viable bacteria, as evidenced by negative blood cultures. It has been proposed that this material represents solubilized endotoxin from either the organisms in the gastrointestinal tract or a gram-negative bacterial infection of certain soft tissues (9).

The present investigations were undertaken to determine whether free endotoxin is the result of metabolic overproduction of lipopolysaccharide or whether it can be attributed simply to solubilization in a liquid environment. Additionally, attempts were made to determine what portion of the LPS molecule is represented by free endotoxin.

Materials and Methods. Limulus assay. Endotoxin assays were performed using methods previously described (14). Lysate used in this study could detect as little as 1 ng/ml of *Escherichia coli* 055:B5 endotoxin (Boivin extract, Difco). Quantitation of endotoxin activity was accomplished by assay of appropriate dilutions of the material being tested in pyrogen free 0.85% saline (Travenol).

Measurement of bound and free endotoxin. A strain of *Escherichia coli* designated G-R isolated from clinical material was grown with and without agitation in a 37° water bath in both brain heart infusion broth (BBL) and a minimal medium containing 0.5% glucose and mineral salts. The mineral salts solution had the following formula:

$(NH_4)_2HPO_4$	1.00 g
K_2HPO_4	1.00 g
NaCl	5.00 g
$MgSO_4 \cdot 7\,H_2O$	0.20 g
Pyrogen-free distilled water (Travenol)	1000 ml

E. coli G-R resting cells were prepared by first growing the organism in brain heart infusion broth or on brain heart infusion agar, then resuspending the cells in 0.85% NaCl (Travenol). Resting cells were then agitated at 37° in the saline suspending medium.

Viable cells were quantitated by replicate plate counts on brain heart infusion agar by the spread plate technique of inoculation. The Limulus assay was used to measure the endotoxin content of both the whole culture and the culture filtrate obtained by passage through a 0.22 μm membrane filter (Millipore). As an index of cellular lysis, the nucleic acid content of the culture filtrate was estimated by optical density readings at 260 nm using a Beckman DB-G Grating Spectrophotometer.

Chemically extracted endotoxin. Aqueous phenol extracts of *Pseudomonas aeruginosa* Verder and Evans Group II, serotype 1369 (kindly supplied by Dr. J. A. Bass, Department of Microbiology, The University of Texas Health Science Center at San Antonio), and *E. coli* G-R were prepared by the method of Selzer (15). An additional extract was prepared from the supernatant fluid of a suspension of *E. coli* G-R cells in pyrogen-free distilled water allowed to stand for 24 hr at room temperature. This supernatant LPS was then chemically extracted in exactly the same manner as the cell paste.

Preparation of lipid A and polysaccharide. Lipid A and polysaccharide were prepared from *E. coli* 055:B5 (Difco) and *Pseudomonas aeruginosa* phenol extracted endotoxins. The dried lipopolysaccharide was suspended in 1% glacial acetic acid at a concentration of 10 mg/ml (16). This suspension was heated at 100° for 1 hr, cooled to 5°, and centrifuged at 500*g* for 15 min to collect the lipid A precipitate. Lipid A was washed twice by suspension in pyrogen-free distilled water followed by recentrifugation. The polysaccharide supernatant solution was dialyzed against repeated changes of Pyrogen-free distilled water at 4° for 2 days. Both preparations were then lyophilized in pyrogen-free glass vials.

Comparison of endotoxins from smooth and rough bacterial strains. Three R-form mutants and their parent smooth strains were compared for whole culture and free endotoxin content. The strains, generously supplied by Dr. H. Y. Whang, Departments of Microbiology and Pediatrics, State University of New York at Buffalo included *Escherichia coli* F576 ($O8^-$:$K42^-$) having *E. coli* core type R-2, and its parent smooth

strain, *E. coli* A295b (08^+:$K42^+$); *Shigella boydii* 3140 (type 3^-) having *E. coli* core type R-1, and its parent smooth strain *S. boydii* 3141 (type 3^+); and *Salmonella minnesota* R597 (RD) having an incomplete *Salmonella* core, and its parent strain *S. minnesota* 1114. These organisms were grown in pyrogen-free 0.5% glucose and mineral salts medium at 37° without agitation. Samples for Limulus assay of whole culture and culture filtrate, and for viable cell counts were obtained at 0, 8, 12 and 24 hr of incubation.

Results. When *Escherichia coli* G-R was grown in brain heart infusion broth with and without agitation, large amounts of endotoxin could be found to accompany growth of the organism as both whole culture and free endotoxin (Fig. 1). Initial endotoxin concentrations under either set of conditions were 32 ng/ml for whole cultures and 16 ng/ml for the culture filtrate. After 24 hr of growth with agitation, these values increased to 300 μg/ml and 200 μg/ml, respectively. The viable cell count increased from 8.0×10^4 cells/ml at time zero to 6.4×10^9 cells/ml after 24 hr. When grown in brain heart infusion broth without agitation, the whole culture endotoxin value was 140 μg/ml whereas free endotoxin content was 100 μg/ml. The viable cell count under these conditions increased only to 1.4×10^9 cells/ml. However, under both conditions approximately a 4 log increase in both whole culture and free endotoxin was observed.

When *E. coli* G-R was grown both with and without agitation in 0.5% glucose and mineral salts medium, a similar large increase in both whole culture and free endotoxin accompanied growth (Fig. 2). After 24 hr incubation, the whole culture endotoxin concentration was 120 μg/ml with 80 μg/ml free endotoxin from a viable cell count of 1.1×10^9 organisms/ml. An endotoxin content of 60 μg/ml and 32 μg/ml, respectively, from 4.8×10^9 viable cells/ml could be demonstrated under nonagitated growth conditions. As in the case of brain heart infusion broth, there was a 3–4 log increase in both whole culture and free endotoxin associated with growth of the culture.

When *E. coli* G-R resting cells previously growth either in brain heart infusion broth or on brain heart infusion agar were agitated in 0.85% sodium chloride for 3 hr, a modest but definite increase in free endotoxin could be demonstrated (Fig. 3). The viable cell count and the optical density of the filtrate at 260 nm did not change significantly during the period of the experiment.

Limulus activity of chemically purified solubilized (or free) endotoxin and bound (or cell associated) endotoxin is shown in

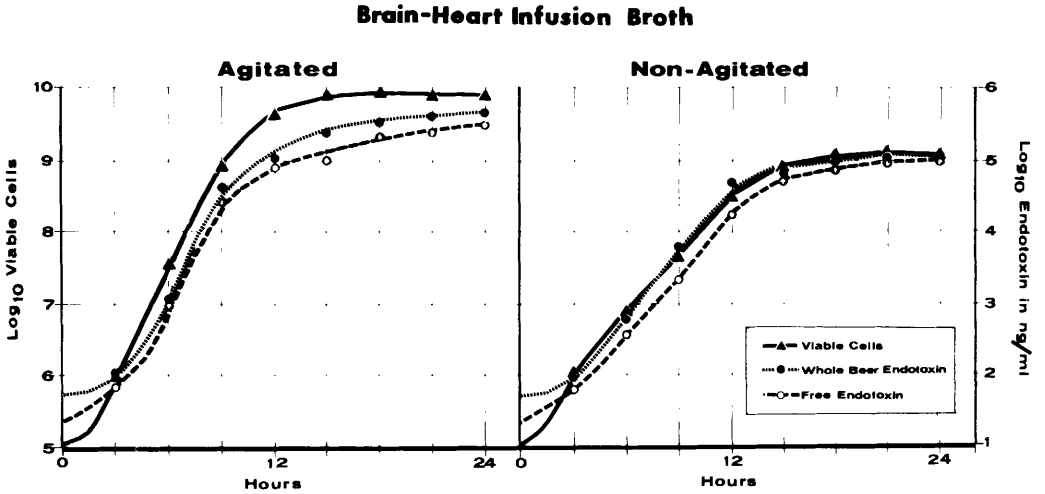

FIG. 1. Whole culture and free endotoxin levels in cultures of *E. coli* G-R cells grown at 37° in brain heart infusion broth with and without agitation.

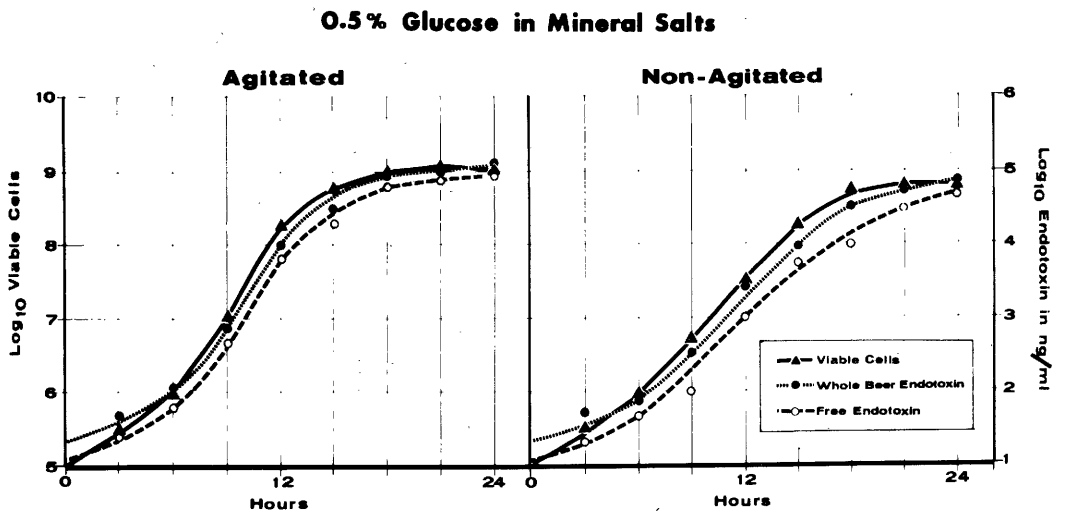

FIG. 2. Whole culture and free endotoxin levels in cultures of *E. coli* G-R cells grown at 37° in 0.5% glucose and mineral salt medium with and without agitation.

Table I. Following extraction by the aqueous phenol method, the activity of bound and free endotoxin appeared to be almost identical.

Results of Limulus tests on lipid A and polysaccharide fractions are reported in Table II. Although lipid A demonstrated somewhat greater Limulus activity than did the polysaccharide fraction, both showed markedly reduced values compared to the parent LPS.

Measurement of whole culture and free endotoxin of R-strains and their parent smooth strains demonstrated that sizable amounts of free endotoxin are produced by R-strains (Table III). A three-log increase in both whole culture and free endotoxin was observed with each R-strain. However, the parent smooth strains in general produced approximately twice as much free endotoxin as the R-strains during the growth period.

Discussion. The Limulus assay has been shown by several investigators to be a rapid and reproducible method for detecting and quantitating minute amounts of endotoxin (11, 13, 14). This method appears to measure the biologic activity of endotoxin with-

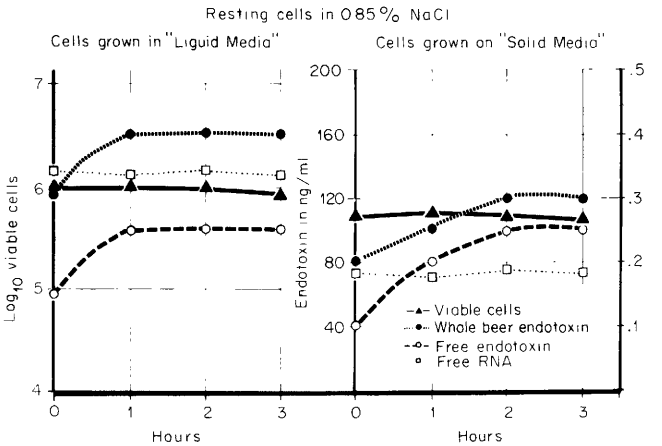

FIG. 3. Solubilization of endotoxin from *E. coli* G-R resting cells grown either in brain heart infusion broth or on brain heart infusion agar.

TABLE I. Comparative Limulus Activity of Bound and Free Endotoxin Obtained from *E. coli* G-R.

Preparation	Limulus activity (ng/ml)			
	100	10	1	0.1
Bound endotoxin*	4+	4+	4+	1+
Free endotoxin**	4+	4+	3+	1+

* Phenol extract of cell paste.

** Phenol extract of supernatant fluids as described in text.

out many of the problems associated with bioassays. Although the specificity of the Limulus assay has been recently questioned (17), the findings of that study have not been confirmed, and in fact are in contradiction with two previous investigations (8, 13). Therefore, the Limulus assay appears to be the most practical method presently available for the detection of endotoxin.

In the present study, the Limulus assay has allowed measurement of naturally occurring endotoxin activity as a function of growth of cultures of gram negative bacilli. Both the endotoxic activity of the entire culture contents and the culture filtrate were measured. "Free" endotoxin was defined as the activity of the cell-free filtrate obtained by passage of the culture through a membrane filter. The difference in endotoxic activity between the whole culture and filtrate measurements could then be described as persistent cell associated or "bound" endotoxin assuming that a significant amount of LPS was not retained by the filter. These results demonstrate that the endotoxic activity of LPS remaining on the surface of intact cells is readily measurable by the Limulus assay.

The findings that the presence of free endotoxin closely accompanied growth of the cells in either enriched or minimal media with or without agitation indicates that luxuriant growth conditions are not a necessity for production of free endotoxin. Results of the resting cell experiment indicated that there was an increase in free endotoxin in the absence of either measurable growth or lysis of the cells. The increase in free endotoxin from the resting cells appears to be due to increased solubilization or shedding of preexisting cell wall material, and is probably not a consequence of metabolic overproduction of this material, nor of cellular lysis. Support for these conclusions is provided by the recent findings that endotoxin containing cell wall blebs are released from *meningococci* during log phase growth in either defined or complex, liquid or solid media (18). Therefore, free endotoxin production appears to occur with *meningococci,* much as we have described with *E. coli* in the present experiments.

The questions regarding which portion of the LPS is represented by free endotoxin and what degree of endotoxicity it possesses then arise. It would seem likely that the oligosaccharide 0-side chains of the LPS would be the most readily solubilizable portion of the LPS molecule, and that free endotoxin might be composed largely of this terminal polysaccharide, rather than the

TABLE II. Limulus Assay Activity of Lipid A and Polysaccharide Derived by Acetic Acid Hydrolysis of LPS.

Sample no.	Organism	Quantity required to produce positive Limulus test		
		Parent LPS	Lipid A	Polysaccharide
1.	*P. aeruginosa*	1 ng/ml	60 ng/ml	100 ng/ml
2.	*P. aeruginosa*	1 ng/ml	100 ng/ml	100 ng/ml
3.	*P. aeruginosa*	1 ng/ml	N.D.*	80 ng/ml
4.	*E. coli* G-R	1 ng/ml	50 ng/ml	200 ng/ml
5.	*E. coli* G-R	1 ng/ml	50 ng/ml	200 ng/ml
6.	*E. coli* G-R	1 ng/ml	60 ng/ml	100 ng/ml

* Insufficient product formed for testing purposes.

TABLE III. Measurement of Bound and Free Endotoxin Produced by R-Strains and Their Parent Smooth Strains While Growing in 0.5% Glucose and Mineral Salts Without Agitation.

Sample time	Organisms*	Viable cells/ml	Endotoxin in whole beer (μg/ml)	Endotoxin in culture filtrate** (μg/ml)
0 hr.	*E. coli* A295b	4.2×10^3	0.010	0.001
	E. coli F576	9.8×10^2	0.008	0.001
	S. minnesota 1114	4.0×10^3	0.008	0.001
	S. minnesota R597	3.2×10^3	0.008	neg.
	S. boydii 3141	5.2×10^3	0.004	neg.
	S. boydii 3140	4.0×10^3	0.004	neg.
8 hr.	*E. coli* A295b	6.0×10^5	1	0.4
	E. coli F576	2.0×10^5	0.8	0.2
	S. minnesota 1114	8.6×10^5	1	0.4
	S. minnesota R597	7.2×10^5	1	0.2
	S. boydii 4131	7.3×10^5	0.8	0.2
	S. boydii 3140	5.0×10^5	0.8	0.2
16 hr.	*E. coli* A295b	1.16×10^7	100	40
	E. coli F576	1.41×10^7	40	10
	S. minnesota 1114	1.46×10^7	40	10
	S. minnesota R597	3.34×10^7	40	10
	S. boydii 3141	5.7×10^6	20	20
	S. boydii 3140	8.1×10^6	20	10
24 hr.	*E. coli* A295b	3.9×10^6	100	40
	E. coli F576	5.8×10^6	40	20
	S. minnesota 1114	7.0×10^6	40	20
	S. minnesota R597	9.7×10^6	40	10
	S. boydii 3141	6.0×10^5	40	20
	S. boydii 3140	1.1×10^6	40	10

* R-strains: *E. coli* F576, *S. minnesota* R597, *S. boydii* 3140; S-strains: *E. coli* A295b, *S. minnesota* 1114, *S. boydii* 3141.

** Culture filtrate obtained by passage through 0.22 μm Millipore filter.

lipid A-KDO-oligosaccharide core material. If endotoxicity of LPS is contributed mainly by the lipid A-core region, free endotoxin might therefore be expected to demonstrate less endotoxin activity per unit weight. However, measurement of Limulus activity following the same chemical extraction procedures, indicates that bound and free endotoxin appear to have essentially the same endotoxic activity.

Since both lipid A and polysaccharide fractions derived by acid hydrolysis were of markedly lower toxicity than the parent LPS (Table II), it would appear reasonable to assume that free endotoxin is not merely a side chain or fragment but is in fact of the same general structure and composition as bound LPS. Undegraded LPS demonstrated 50–100 times more reactivity than lipid A, and 80–200 times more reactivity than polysaccharide alone. This marked decrease in endotoxic activity as measured by the Limulus assay agrees with previous findings of investigators using other methods of endotoxin assay (5). However, it has been suggested the reduced biologic activity of lipid A is due to decreased solubility in an aqueous environment. Certain investigators (19, 20) have demonstrated increased solubility and toxicity of lipid A when complexed with bovine serum albumin (BSA). Since it has been shown that BSA preparations may frequently be contaminated with large amounts of endotoxin (21), the increase in activity of lipid A suspended in BSA may be open to question.

In an effort to determine more precisely the source of free endotoxin, measurements of bound and free endotoxin were performed on R-form mutants of 3 gram-nega-

tive organisms and the smooth strains from which they were derived. The results presented in Table III indicate that R-forms, which are almost totally devoid of 0-side chain polysaccharides, produce ample amounts of free endotoxin. However, the total amounts of free endotoxin produced by the R-strains were generally about one-half the amounts produced by their parent smooth strains. These findings lend further support to the concept that free endotoxin is not composed simply of easily solubilizable 0-side chain material, but rather consists of core lipopolysaccharide material that is in some fashion released into a liquid environment. However, because of its solubility and close association with LPS core, 0-antigen side chain material must certainly accompany free endotoxin in smooth bacterial strains.

The concept that viable gram-negative bacterial cells do not release endotoxin unless autolysis or disruption occurs should be replaced with the newer concept that viable cells readily release endotoxin by some form of solubilization. This does not preclude the possibility that lysis may release additional amounts of endotoxin. Therefore, it is possible that *in vivo* free endotoxin occurs either from simple solubilization or from immune lysis of bacterial cells.

These findings also suggest that a coating of bound endotoxin persists on the cell surface. Therefore, quantitative measurement of bound or whole culture endotoxin may be employed as a method of obtaining an approximate quantitation of gram-negative bacilli per volume of a particular fluid (10). If large amounts of free endotoxin are suspected, the component contributed by bound endotoxin can be determined by comparing endotoxin levels before and after passage through a membrane filter.

Summary. The Limulus assay was used to measure activities of naturally occurring and chemically extracted endotoxins and endotoxin fractions. Large amounts of both bound and free endotoxin were detected from gram-negative bacterial cells grown in either enriched or minimal media, and from resting cells. Following purification by phenol extraction, free endotoxin evidenced a similar level of activity to that of bound endotoxin. Lipid A and polysaccharide prepared by hydrolysis of endotoxin demonstrated reduced Limulus activity. However, R-strains from three species of gram-negative bacilli produced large amounts of free endotoxin. These results indicate that free endotoxin is part of the LPS that is readily released in a liquid environment, and probably is composed of lipid A and core material. In addition, a relatively constant amount of endotoxin remains bound on the cell surface, allowing an approximate quantitation of viable bacilli in fluids using the Limulus assay.

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