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Influence of Fine Structure of Lipid A on *Limulus* Amebocyte Lysate Clotting and Toxic Activities

KUNI TAKAYAMA,[1,2] NILOFER QURESHI,[1] CHRISTIAN R. H. RAETZ,[3] EDGAR RIBI,[4]* JON PETERSON,[4] JOHN L. CANTRELL,[4] FREDERICK C. PEARSON,[5] JEFFRY WIGGINS,[5] AND ARTHUR G. JOHNSON[6]

Mycobacteriology Research Laboratory, William S. Middleton Memorial Veterans Hospital, Madison, Wisconsin 53705[1]; The Institute for Enzyme Research[2] and Department of Biochemistry, College of Agricultural and Life Sciences,[3] University of Wisconsin, Madison, Wisconsin 53705; Ribi ImmunoChem Research, Inc., Hamilton, Montana 59840[4]; Travenol Laboratories, Inc., Morton Grove, Illinois, 60053[5]; and Department of Medical Microbiology and Immunology, University of Minnesota, Duluth, Minnesota 55812[6]

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We examined the relationship between the fine structure of lipid A and the toxicity of endotoxin or lipopolysaccharides as measured by the *Limulus* amebocyte lysate (LAL), rabbit pyrogenicity, chicken embryo lethal dose, and dermal Shwartzman reaction tests. Lipid A and lipid A-like compounds obtained from deep-rough mutants of *Salmonella* spp. and *Escherichia coli* had a wide range of structural variations. These compounds included native lipopolysaccharides, diphosphoryl and monophosphoryl lipid A's, and lipid X (a monosaccharide). The LAL test was positive for all lipids tested with lysates from Travenol Laboratories and from Associates of Cape Cod (2.9×10^3 to 2.6×10^7 endotoxin units per mg), except for *O*-deacylated and dephosphorylated lipid X, which were negative. The Mallinckrodt lysate gave negative tests for lipid X. In the rabbit pyrogenicity and chicken embryo lethal dose tests, only native lipopolysaccharide and diphosphoryl lipid A's were judged toxic. The Shwartzman reaction was positive for a specific purified diphosphoryl lipid A (thin-layer chromatography-3 fraction) but negative for the purified monophosphoryl lipid A (also a thin-layer chromatography-3 fraction). These results show that the LAL test is not a valid measure of all parameters of toxicity of a lipid A or lipid A-like compound and can yield false-positive results. However, these findings are not in conflict with the widespread use of the LAL assay for pyrogens in the pharmaceutical industry since a good correlation exists between LAL results and pyrogenicity when undegraded endotoxin is evaluated in parallel assays.

Takayama et al. (35, 36; K. Takayama, N. Qureshi, E. Ribi, and J. L. Cantrell, Rev. Infect. Dis., in press) used highly purified and well-characterized samples of precursors and derivatives of bacterial lipopolysaccharides (LPS) to study the relationship between the chemical structure of lipid A and its in vivo toxicity. These studies included the use of tests to observe direct lethality to animals and of the very sensitive chicken embryo lethal dose ($CELD_{50}$) test. Some preliminary *Limulus* amebocyte lysate (LAL) tests were also performed on these lipids. Results suggested a multiple structural requirement for toxicity of lipid A. However, two other standard tests for in vivo toxicity of endotoxin (fever in rabbits and dermal Shwartzman reaction) were omitted (7).

The basis of the *Limulus* test is an endotoxin-induced coagulation reaction with an amebocyte lysate which was first reported by Levin and Bang (1, 13, 14). Subsequent studies by Levin and co-workers established that the LAL assay could detect as little as 5 ng of circulating endotoxin per ml in plasma, thereby suggesting the potential utility of this assay in the detection of endotoxemia (15). Although the LAL assay has been proven clinically accurate in the detection of gram-negative bacteria (8) and meningitis (9), the most widespread use of the test has been in the pharmaceutical industry for in-process and end product pyrogen testing. Numerous products have been successfully evaluated for pyrogenicity by the LAL assay, including medical devices (25, 40), large-volume parenterals (25), small-volume parenterals (16), plasma fractions (23), and radiopharmaceuticals (2).

* Corresponding author.

Although the LAL assay is exquisitely sensitive to picogram amounts of endotoxin and has been sanctioned by the Food and Drug Administration and the United States Pharmacopoeia, the test must be properly controlled to rule out false-positive and false-negative reactions (21, 37). False-positive LAL reactions have been induced by cellulose-derived material from hemodialysis membranes as well as other similar polysaccharide materials (18, 31, 42; F. C. Pearson, J. Bohon, W. Lee, G. Bruszer, M. Sagona, R. Dawe, G. Jacubowski, D. Morrison, and C. Dinarello, Int. Soc. Artif. Organs, in press).

When the LAL assay is used for basic biological studies, the important question arises concerning how coagulation in a biological system derived from a horseshoe crab could be related to toxicity in mammals. Is the *Limulus* test a good measure of the presence of lipid A or the toxicity of a lipid A preparation or both?

We have examined the question of how the structure of lipid A affects the outcome of these various toxicity tests. Our results suggest that although a direct relationship can be shown between the standard in vivo assays for toxicity (such as rabbit pyrogenicity, $CELD_{50}$, and dermal Shwartzman reaction) of lipid A and lipid A-like structures, a comparable relationship does not exist for the LAL assay.

MATERIALS AND METHODS

Preparation of LPS, lipid A, and lipid X. LPS from *Salmonella typhimurium* G30/C21 and *Salmonella minnesota* R595 was prepared by the method of Galanos et al. (6) as modified by Takayama et al. (36). Diphosphoryl lipid A's were prepared by the pH 4.5 treatment according to Rosner et al. (30) and purified by thin-layer chromatography as

previously described (35; Takayama et al., in press). Monophosphoryl lipid A's and purified components were prepared as described by Qureshi et al. (27). Lipid X was prepared from *Escherichia coli* MN7 by the method of Takayama et al. (34).

Biological assays. Rabbit pyrogenicity assay and LAL assay with the Travenol Laboratories lysate (gel clot method; Travenol Laboratories, Inc., Morton Grove, Ill.) were performed by the method of Weary et al. (41). The LAL kits were obtained from Associates of Cape Cod, Inc., Woods Hole, Mass. (Pyrotell) and Mallinckrodt, Inc., St. Louis, Mo. (Pyrogent and Color Lysate Chemistry). The procedure for the $CELD_{50}$ assay was described by Milner and Finkelstein (17). Testing for the capacity of lipid A's to prepare for the local Shwartzman reaction was done by intradermal injection of purified glycolipid into 8- to 10-week-old New Zealand white rabbits. The provoking intravenous challenge consisted of 40 μg of a standard reference endotoxin or diphosphoryl or monophosphoryl compounds administered 18 h after preparation. Readings were made at 6, 24, and 48 h.

For the *Limulus* assays, a stock solution of glycolipid was dissolved in pyrogen-free water (American McGaw, Irvine, Calif., and Travenol Laboratories) containing up to 0.5% triethylamine to a concentration of 1.0 mg/ml. All preparations used appeared clear to opalescent in nature. A control triethylamine solution was included in the assay. The stock solutions were diluted with pyrogen-free water and used in the LAL assays. Similar solutions were made for the $CELD_{50}$, rabbit pyrogen assay, and Shwartzman reaction.

RESULTS

LAL activity. LAL assays of endotoxin and both unfractionated and purified monophosphoryl and diphosphoryl lipid A's all gave values in the range of 1.1×10^5 to 2.6×10^7 endotoxin units (EU)/mg with the Travenol Laboratories lysate (Table 1). Similarly, the Associates of Cape Cod lysate by the gel clot method gave 2.8×10^5 and 2.9×10^6 EU/mg, the Mallinckrodt lysate by the gel clot method gave 2.0×10^5 and 8.5×10^5 EU/mg, and the Mallinckrodt lysate by the chromogenic method gave 3.2×10^6 and 7.5×10^6 EU/mg with the mono- and diphosphoryl lipid A's, respectively (Table 2). This is consistent with the variations in sensitivity among different commercial LAL reagents of up to 100-fold as observed by Wachtel and Tsuji (39). The monosaccharide lipid X gave positive LAL tests with both the Travenol Laboratories and Associates of Cape Cod lysates (9.5×10^4 and 2.9×10^3 EU/mg, respectively). These values compare favorably with that for the standard endotoxin from *E. coli* O55:B5 for indicating toxicity. The Mallinckrodt lysate, however, gave negative results with lipid X (1.2 and 7.0 EU/mg by the gel clot and chromogenic methods, respectively) (Table 2). When lipid X was either *O*-deacylated or dephosphorylated, there was a significant reduction in LAL values, suggesting that the ester group at the 3-position of the sugar is important for activity (Fig. 1). The phosphate group also appears to be required for LAL activity, although part of the decreased activity might be due to the difficulty in obtaining well-dispersed samples to test.

Rabbit pyrogenicity and $CELD_{50}$ tests. Both endotoxin and diphosphoryl lipid A, regardless of whether they were purified or not, exhibited high pyrogenic activity (Table 1). Activity ranged from a high value of 0.0005 to 0.001 μg/kg to a low value of 0.012 μg/kg. It was clear that the diphosphoryl lipid A's were extremely pyrogenic and that these results correlated well with the high toxicity revealed by the $CELD_{50}$ test. When monophosphoryl lipid A's and lipid X were similarly tested, they showed very low pyrogenicity, with values ranging from 5 to 10 μg/kg. The corresponding $CELD_{50}$ values were all 20 μg for this latter set, which is consistent with previous results (Takayama et al., in press). Although pyrogenicity and $CELD_{50}$ values suggested a lack of toxicity, LAL tests were positive for both monophosphoryl lipid A's and lipid X (only when Travenol Laboratories or Associates of Cape Cod lysates were used). Thus, it was clear that a positive LAL result is not a good measure of toxicity by pyrogenicity or $CELD_{50}$ criteria when measuring the activity of endotoxin precursor(s) or degraded endotoxins. This argument can be extended to lethality in animals as previously described (35).

Dermal Shwartzman reaction in rabbits. The dermal Shwartzman reaction is another measure of the in vivo toxicity of endotoxin. The dose-response tabulation in Table 3 shows that the hemorrhagic reaction was very intense at 20 μg for diphosphoryl lipid A's. A much lower level of about 0.5 μg was required to obtain a low hemorrhagic response. For the monophosphoryl lipid A, a relatively high dosage level of 100 μg occasionally gave weak transient hemorrhagic reactions approximately one-tenth of the size of that seen with 5 or 20 μg of the diphosphoryl compound. The monophosphoryl compound was not titrated beyond 100 μg. These results were similar regardless of whether unfractionated or purified lipid A was used. Figure 2 vividly illustrates the difference between the mono- and diphosphoryl lipid A's. From these results it is clear that the monophosphoryl

TABLE 1. Effect of structure of LPS, lipid A, and lipid X on some of the commonly used toxicity measurements

Compound	Source[a]	*Limulus* activity[b] (EU/mg)	Rabbit pyrogenicity APD_{50}[c] (μg/kg)	$CELD_{50}$ (μg)
Endotoxin	A	2.6×10^7	0.0001–0.0003	0.0031
Endotoxin	B	1.3×10^7	0.0002–0.0007	0.0034
Endotoxin	C	5.0×10^6	0.0015	ND[d]
Diphosphoryl lipid A (unfractionated)	A	2.6×10^6	0.0005–0.001	0.0088
Diphosphoryl lipid A (purified, TLC-3)	A	8.0×10^5	0.012	0.0064
Monophosphoryl lipid A (unfractionated)	B	1.0×10^6	2–5	6.7
Monophosphoryl lipid A (purified, TLC-3)	B	1.1×10^5	>10	>20
Lipid X	D	9.5×10^4	>10	>20
Lipid X (*O*-deacylated)	D	1.4×10^2	>10	>20
Lipid X (dephosphorylated)	D	3.2×10^1	>10	>20

[a] Source: A, *S. typhimurium* G30/C21; B, *S. minnesota* R595; C, *E. coli* O55:B5 (Travenol Laboratories, Inc.); D, *E. coli* MN7.

[b] EU, Defined as the potency of 0.2 ng of an EC-2 reference standard endotoxin (gel clot method). Source of lysate, Travenol Laboratories, Inc. (sensitivity, 0.13 EU/ml).

[c] The approximate dose necessary to cause a febrile response of >0.46°C in 50% of a test population.

[d] ND, Not done.

TABLE 2. Influence of structure of lipid A on LAL activity of lysates from two sources using two different methods

Compound[a]	*Limulus* activity (EU/mg) using lysates from:		
	Associates of Cape Cod (gel clot)[b]	Mallinckrodt	
		Gel clot[c]	Chromogenic[d]
Diphosphoryl lipid A (unfractionated)	2.9×10^6	8.5×10^5	7.5×10^6
Monophosphoryl lipid A (unfractionated)	2.8×10^5	2.0×10^5	3.2×10^6
Lipid X	2.9×10^3	1.2	7.0

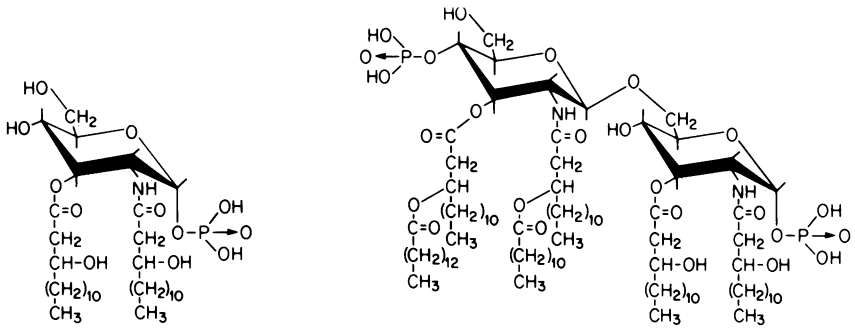
[a] Source of compounds are indicated in Table 1, footnote *a*.
[b] Sensitivity, 0.02 EU/ml.
[c] Sensitivity, 0.06 EU/ml.
[d] Sensitivity, 0.1 to 1.0 EU/ml.

lipid A is at least 200 times less active than the diphosphoryl lipid A in eliciting the dermal Shwartzman reaction.

DISCUSSION

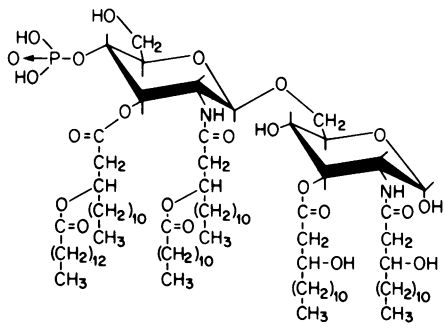
Crude lipid A preparations consist primarily of unhydrolyzed LPS, diphosphoryl lipid A's, monophosphoryl lipid A's, and free fatty acids (35, 36; Takayama et al., in press). Depending on the conditions of acid hydrolysis, the predominant product can be either a diphosphoryl lipid A (thin-layer chromatography [TLC]-3 analog of diphosphoryl lipid A, a product of hydrolysis of native endotoxin in sodium acetate, pH 4.5) (36; Takayama et al., in press) or a monophosphoryl lipid A (TLC-3 analog of monophosphoryl lipid A, a product of native endotoxin in 0.1 N HCl) (27). These products have now been isolated and completely characterized (26, 27, 32); their structures are shown in Fig. 1. Structures of native endotoxins from the rough mutants *S. typhimurium* G30/C21 and *S. minnesota* R595 would be represented by the diphosphoryl lipid A (TLC-3 fraction) to which are attached the 2-keto-3-deoxyoctulosonic acid groups at the 6′ position. Phosphorylethanolamine could be on the phosphate at the reducing end, and 4-amino-4-deoxy-L-arabinose might be on the phosphate in the 4′ position (12, 20, 38). The latter two components are probably substituted randomly. Additionally, the LPS from the R595 strain contains an extra palmitoyl group (43).

Another useful lipid A-like compound is lipid X, isolated from *E. coli* MN7 by Nishijima and Raetz (22) and recently characterized by Takayama et al. (34). Its structure is also shown in Fig. 1. Lipid X is a glucosamine monosaccharide containing two β-hydroxymyristic acid residues and one phosphate group. It appears to be a precursor for the reducing-end subunit of lipid A. Both lipids X and Y (33) and their derivatives were recently used by Raetz et al. (28) to show that the minimal structural requirement for B-lymphocyte activation by lipid A is the reducing-end subunit. The activity required the presence of the 3-*O*-acyl group. Lipids are now available which have interesting variations in their structure, allowing us to examine how fine structure influences certain biological activities (Fig. 1). These highly purified and completely characterized lipids, along with native LPS from the rough mutants of *Salmonella* strains, were used to study structure versus LAL activity, pyroge-



Lipid x

Diphosphoryl lipid A, TLC-3



Monophosphoryl lipid A, TLC-3

FIG. 1. Structures of lipid X and specific lipid A's which were used in this study. Note that lipid X is structurally identical to the reducing-end subunit of diphosphoryl lipid A (TLC-3 fraction). Although both mono- and diphosphoryl lipid A's (TLC-3 fraction) are the most prominent components in their respective preparations, other forms with lower fatty acid content are also present in a mixture (27, 36).

TABLE 3. Effect of dosage levels of mono- and diphosphoryl lipid A on the local Shwartzman reaction

Compound	Preparative dose (μg)	Average hemorrhage size (cm)[a]
Diphosphoryl lipid A (unfractionated)	20	2 by 2.5
	5	2 by 1.5
	0.5	0.5 by 0.5
	0.1	Tr
Diphosphoryl lipid A (TLC-3 fraction)	20	2 by 2.5
	5	2 by 2
	0.5	0.5 by 0.5
	0.1	Tr
Monophosphoryl lipid A (TLC-3 fraction)	100	0.3 by 0.3
	20	Negative

[a] Twelve 10-week-old female New Zealand white rabbits were prepared and provoked with 40 μg of reference or diphosphoryl endotoxin 18 h after intradermal preparation. Lesions were read at 6 and 24 h.

nicity, and local Shwartzman reaction in rabbits, and lethality in chicken embryos.

Although our results reported here with native LPS are in agreement with those obtained by Elin et al. (4), who reported a significant correlation between pyrogenicity and LAL activity, their study lacked the important structural variations in the lipid A moiety of the 17 LPS examined. Wachtel and Tsuji (39) found that LAL and rabbit pyrogen test data correlated well only when relatively pure and undegraded forms of endotoxin were examined. Poor correlation was found when partially degraded endotoxins were compared, suggesting that the fine structure of lipid A is important. Additional studies by Pearson et al. (24) suggested that "environmental" endotoxins are 2- to 10-fold more reactive with the LAL assay than predicted by the rabbit pyrogen assay.

Our results are not in agreement with those of Kiso et al. (10, 11), who reported that organically synthesized monosaccharide lipid A derivatives lacking *O*-acyl and phosphate groups possessed LAL gelation activity comparable to that of reference LPS from *E. coli* O55:B5. These compounds would be structurally similar to *O*-deacylated and dephosphorylated lipid X. Both synthetic products and naturally occurring LPS were active at the lowest concentration reported (10^{-5} μg/ml). Although we cannot offer a clear explanation for this discrepancy, it might be due to the omission in their study of the LAL geiation endpoints or to the sensitivity of the LAL preparation used, since commercial formulations differ widely and are standardized to purified LPS.

The Travenol Laboratories and Associates of Cape Cod lysates showed a difference in LAL activity between lipids A and X of 1 and 2 orders of magnitude, respectively. These results could represent false-positive responses which are similar to those of cellulosic materials (Pearson et al., in press). The Mallinckrodt lysate showed a more pronounced difference of >5 orders of magnitude in LAL activity between the two lipids. This negative result could be explained on the basis that an endogenous inhibitor is present in the lysate which prevents false-positive results (Pearson et al., in press). Since lipid X is more readily soluble in aqueous medium (containing triethylamine) than lipid A, it is unlikely that this low LAL activity (or lack of it) was due to low solubility.

On the basis of the structures of the lipid A's and lipid X, we can now define the structure-to-LAL activity relationship. With the Travenol Laboratories or Associates of Cape Cod lysate, LAL activity requires either a mono- or a disaccharide containing at least one phosphate group. The Mallinckrodt lysate differs from the other two lysates tested in that it is able to differentiate between a mono- and a disaccharide. Low or negative LAL test activity is obtained only after all of the phosphate or ester-linked fatty acids are removed.

The in vivo toxicity-structure relationships of endotoxin and lipid A's follow a different pattern than that for the LAL test. Toxicity appears to require the presence of a disaccharide, two phosphate groups, and at least one ester-linked fatty acid. By this criterion, the monophosphoryl lipid A's and lipid X are essentially nontoxic. Another essential feature for toxicity (as revealed by a previous study) is the presence of an ester of a normal fatty acid in an acyloxyacyl linkage (Takayama et al., in press).

Elin and Wolff (5) reported that several compounds structurally different from bacterial endotoxins, such as polyinosinic acid-polycytidylic acid and certain proteins, are capable of causing a positive LAL test. The findings described here showed that although monophosphoryl lipid A's and lipid X gave positive LAL test results, these compounds were essentially nontoxic according to the several criteria used. This new observation is important because monophosphoryl lipid A is now considered to be a biological response modifier for human use (29), and the LAL assay would not be a valid test to evaluate its immunomodulatory capability.

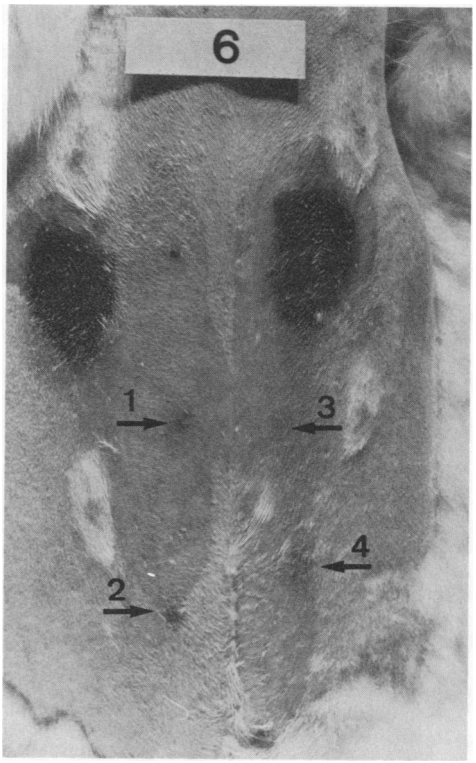


FIG. 2. The dermal Shwartzman reaction in rabbits as elicited by mono- and diphosphoryl lipid A's. The dark spot on the left side was due to injection of 20 μg of unfractionated diphosphoryl lipid A mixture; the dark spot on the right side was due to injection of 20 μg of purified diphosphoryl lipid A (TLC-3 fraction). Other injection points were: 1 and 2, 20 and 100 μg of unfractionated monophosphoryl lipid A mixture, respectively; 3 and 4, 20 and 100 μg of purified monophosphoryl lipid A (TLC-3 fraction), respectively.

The dermal Shwartzman reaction in rabbits is considered to be a realistic in vivo model of endotoxin-caused blood clotting disturbances that characterize gram-negative bacteremia in patients. Under the conditions examined in this study, diphosphoryl lipid A was highly active, whereas monophosphoryl lipid A was essentially inactive in preparing for or provoking the Shwartzman reaction. This observation might be explained on the basis of a mechanism proposed by Morrison and Cochrane (19). They suggested that the phosphate residue of the lipid A segment of endotoxin initiates coagulation by activating Hageman factor XII. Alternatively, the presence or absence of the phosphate group in lipid A might affect the induction of procoagulant activity of monocytes (3). We suggest that the phosphate residue situated specifically at the reducing end of the lipid A molecule is involved in the events that lead to the Shwartzman phenomenon. Further discussion of the structure-function relationship of the Shwartzman reaction would be speculative until tests are performed on specific analogs of lipid A, for example, lipid X. Such studies are in progress.

Both mono- and diphosphoryl lipid A possessed comparable activity when tested in vitro for their ability to induce clotting in the LAL assay. The difference in response of toxic and nontoxic lipid A to the Shwartzman and LAL coagulation test system might be due, in part, to a large difference in the sensitivity of the assays to endotoxin. Since gelation of LAL can be activated by several compounds which are structurally unrelated to endotoxin, it is now clear that there is an inherent difference in the mechanism by which clotting occurs in these two systems. Thus, for example, LAL-positive cellulosic materials (Pearson et al., in press) are unlikely to possess the property needed to prepare rabbit skin for a Shwartzman reaction.

These results are not in conflict with the use of the LAL assay for estimating pyrogenicity of parenteral solutions and medical devices, since these products contain native endotoxin which has not been degraded by chemical means. Further work aimed at elucidating the complexities of LAL gelation is now in progress.

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