JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1991, p. 2477–2483
0095-1137/91/112477-07$02.00/0

Vol. 29, No. 11

Sensitivity of *Limulus* Amebocyte Lysate (LAL) to LAL-Reactive Glucans

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Received 13 June 1991/Accepted 9 August 1991

The sensitivity of *Limulus* amebocyte lysate (LAL) to LAL-reactive glucans (LRGs) and lipid A was tested by using commercially available and experimentally formulated LAL reagents. The glucans included two kinds of β-(1,3)-D-glucans, laminarin and curdlan, and cellulosic material, LAL-reactive material (LAL-RM), extracted from a hollow-fiber (Cuprophan) hemodialyzer. LAL-RM loses its LAL activity when it is digested with cellulase and thus appears to be a β-(1,4)-D-glucan or a mixed glucan containing a substantial proportion of β-(1,4) linkages. All LAL reagents tested were at least 1,000-fold more sensitive to endotoxin than to LRGs. The presence of the surfactant Zwittergent was shown to interfere with reactivity to LRGs; LAL reagents without added Zwittergent reacted more strongly to LRGs than did the same reagents containing Zwittergent. Chloroform extraction of LAL increased the reagents' sensitivity to both endotoxin and LRGs, but it was not responsible for LRG reactivity. The addition of Zwittergent significantly reduced the sensitivity of LAL reagents to lipid A. LAL without the surfactant was equally sensitive to endotoxin and lipid A. Both curdlan and LAL-RM amplified or enhanced the LAL response to endotoxin. Kinetic turbidimetric studies demonstrated that the enhancement was dependent on the glucan concentration.

The *Limulus* amebocyte lysate (LAL) test is extremely sensitive to endotoxin and has been considered to be specific for lipopolysaccharide (LPS). However, a few polymeric forms of glucose have been shown to be LAL reactive when they are present in sufficient quantities, i.e., nanogram to microgram amounts per milliliter. These glucans include β-(1,3)-D-glucans, which are found in the cell walls of fungi, yeasts, and algae (2, 9, 10, 12, 16), and LAL-reactive material (LAL-RM), which is presumed to be a β-(1,4)-D-glucan derived from a hollow-fiber hemodialyzer with a saponified cellulose acetate or Cuprophan membrane (4, 23, 24).

Although β-(1,3)-D-glucans are not pyrogenic, they are of interest because of their varied biological activities and their potential for reacting with LAL reagents in pyrogen testing. They act as immunomodulators and have been considered to be signals for biological defense mechanisms (1, 28). β-(1,3)-D-glucan has been suggested as a radioprotective agent (22). Glucans stimulate the reticuloendothelial system and act as immunological adjuvants (6, 10, 13, 31). They inhibit the growth of tumors (3, 6, 12, 27) and have been reported to augment the toxicity of endotoxic septicemia or septic shock (5, 14).

LAL-RM is antigenic, activates complement by the alternative pathway (24), and stimulates the production of interleukin-1 by monocytes (32). Also, LAL-RM can be detected in the plasma of patients undergoing dialysis. The increased incidence of atherosclerosis in patients undergoing dialysis has been linked to an increase in mitogenic activity in arterial smooth muscle during dialysis with Cuprophan filters (8).

LAL reagents with different formulations may show marked differences in response to LAL-reactive glucans (LRGs). Pyrotell, the LAL reagent from Associates of Cape Cod, Inc. (ACC), reacts to a greater degree with glucans than does Pyrogent, the LAL from Mallinkrodt, Inc. (MAL; St. Louis, Mo.), or Whittaker Bioproducts, Inc. (WB; Walkersville, Md.); (23). Thus, some positive LAL reactions may be due to LRGs or a combination of LRGs and endotoxin but not to endotoxin alone. A first step in defining the problem of the reactivities of LAL reagents to LRGs involves assessment of the differences and the reasons for these differences.

LAL reagents are formulated to increase sensitivity to LPS as well as to provide stability and reproducibility. It has long been known that divalent cations increase the sensitivity of the LAL reagent (29). Extraction of the LAL reagent with chloroform also increases sensitivity and is a patented method of ACC (29a). The MAL reagent, Pyrogent, includes divalent cations, a Tris buffer (14a), and Zwittergent, a sulfobetaine amphoteric surfactant added to increase sensitivity to endotoxin (5a). Pyrogent is now manufactured by WB under rights and technology acquired from MAL.

In this study, we attempted to assess the reactivities of various LAL reagents to LRGs and to establish the differences which account for their reactivities with glucans. Several experimental LAL reagents were prepared. These clarified the different results obtained when glucans and glucan-containing mixtures were analyzed by using LAL from different manufacturers.

We also carried out enzyme digestion experiments to establish the difference between β-(1,3)-D-glucan and LAL-RM from cellulose acetate filters.

(Part of this work was presented at the Fifth International Conference on Immunopharmacology, 27 May 1991.)

MATERIALS AND METHODS

All glassware was depyrogenated by dry heat for a minimum of 3 h at 180°C. LAL reagent water was prepared by steam distillation (17) and was confirmed to have less than 0.001 endotoxin unit (EU)/ml (0.01 pg/ml) by the LAL test.

LAL reagents. Pyrotell (lots 99-31-375, 42-18-460, 42-24-466; ACC) and Pyrogent lot N181A (MAL) and lot 8L2340 (WB) were used for gel-clot assays. Lot 42-13-455T (ACC) was used in the kinetic turbidimetric method. The endotoxin

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TABLE 1. Sensitivity of commercial and experimental LAL reagents to endotoxin, lipid A, laminarin, curdlan, and LAL-RM

LAL type and source	Sensitivity (pg/ml) to[a]:				
	Endotoxin	Lipid A	Laminarin	Curdlan	LAL-RM[b]
Commercial LAL					
ACC	3.125	3.1	2.6×10^3	2.6×10^3	35
WB	6.25	2.5×10^2	1.3×10^6	1.3×10^6	0.0
MAL	12.5	2.0×10^3	1.0×10^6	3.1×10^5	0.0
Experimental LAL					
Raw LAL	1.0×10^3	3.1×10^3	1.0×10^8	6.3×10^4	6.3×10^3
NOCHCL3	25	50	1.0×10^7	3.2×10^4	6.3×10^2

[a] Sensitivity is the minimum concentration to give a positive gel-clot test. The initial concentrations of laminarin, curdlan, and lipid A were 1 mg/ml.
[b] LAL-RM was an aqueous extract with no verifiable concentration. An arbitrary initial concentration of 100 ng/ml was used for these data.

standards used were phenol extractions of *Escherichia coli* O113, lots 46 and 49, and contained no fillers (ACC).

Experimental LAL reagents. For raw unformulated LAL, lysates of *Limulus* amebocytes were stored at 4°C. For lot NOCHCL3, Pyrotell was prepared from raw lysate by the specifications of Sullivan and Watson (29a), but without chloroform extraction.

For lot VII-82-0, the LAL reagent was prepared as described in the UK patent (14a), i.e., with a buffer containing 0.02 M Tris maleate (pH 7.5), 0.05 M magnesium chloride, 0.008 M strontium chloride, 0.02 M calcium chloride, and 0.1% lactose, but with no Zwittergent. Lot VII-82-Z was exactly the same as lot VII-82-0, except that 0.02% Zwittergent 3-14 detergent (Calbiochem, San Diego, Calif.) was added to the reagent.

Glucan preparations. Laminarin isolated from *Laminaria digitata* was obtained from Sigma Chemical Co. (St. Louis, Mo.). Highly purified curdlan, a water-insoluble, carboxymethylated powder, was received as a gift from Wako Pure Chemical Industries, Osaka, Japan. It was solubilized with 0.1 N NaOH and was neutralized by the addition of an equal part of 0.2 M Tris HCl buffer (pH 7.4 at 37°C). LAL-RM was extracted from a hollow-fiber Cuprophan filter (catalogue no. 18-514; Cobe Laboratories, Lakewood, Colo.) by recirculating 200 ml of LAL reagent water through the filter for 1 h.

Enzymes. The enzymes laminarinase (EC 3.2.1.6) from a *Penicillium* sp. (Sigma) and cellulase (EC 3.2.1.4) from *Aspergillus niger* (Calbiochem) were reconstituted in 0.1 M sodium acetate buffer (pH 5.2). Substrates were digested at 37°C for 1 h by using 1 U of enzyme per ml of substrate. After digestion, free glucose was measured by using the *ortho*-toluidine reagent (Sigma). Because of the low pH, digested samples were assayed for endotoxin by using Pyrotell reconstituted with Pyrosol (0.2 M Tris HCl buffer [pH 7.4]; ACC).

LAL methodology. Kinetic turbidimetric assays were carried out by using the LAL-5000 automatic endotoxin detection system (ACC) (18, 25). Gel-clot assays were carried out according to the instructions of the manufacturer.

RESULTS

Reaction of raw LAL with LRGs. LAL, with no additives (raw LAL), was tested for its reactivity with curdlan, laminarin, LAL-RM, lipid A, and endotoxin. Gelation of this LAL required 1.0 ng of endotoxin per ml, 100 μg of laminarin per ml, 62 ng of curdlan per ml, and 3 ng of lipid A per ml (Table 1). Raw LAL also reacted with LAL-RM. These numbers represent the concentration of LRG or lipid A in the greatest dilution that gave a firm gel (endpoint) in the gel-clot test.

Sensitivities of LAL reagents. Table 1 records the sensitivities of commercial and experimental LAL formulations to endotoxin, LRGs, and lipid A. These data represent the minimum concentration that gave a positive gel-clot endpoint. Lipid A, curdlan, and laminarin were prepared at concentrations of 1 mg/ml. LAL-RM was an aqueous extract of the filter and had no verifiable concentration. An arbitrary value of 100 ng/ml was used as the initial concentration of LAL-RM in order to construct Table 1.

Commercial LAL reagents. The sensitivity of any LAL reagent to LRGs was at least 1,000-fold less than its sensitivity to standard endotoxin. However, the LAL reagent from ACC was more sensitive to LRGs than the LAL reagent from either WB or MAL (Table 1). While this difference was slightly variable when different lots of LAL reagent were used, there was an approximately 200-fold difference between the sensitivity of the ACC LAL to LRGs and that of the MAL or the WB LAL. The sensitivities of all LAL reagents tested except those of MAL and WB were almost the same with lipid A or endotoxin.

Effect of chloroform. Comparison of the data obtained with lot NOCHCL3 LAL (which was not chloroform extracted) and ACC LAL showed that the sensitivity of the ACC LAL reagent to both endotoxin and LRGs was increased by chloroform extraction of the LAL during formulation (Table 1). Sensitivity to endotoxin increased from 25 to 3.125 pg/ml, and sensitivity to laminarin increased from 10,000 to 2.6 ng/ml. The sensitivity to LAL-RM, curdlan, and lipid A was also increased. Alteration of other constituents of LAL reagents such as salts, buffer, lactose, or albumin had no effect on the sensitivity of LAL to LRGs (data not shown).

Effect of Zwittergent. Experimental formulations of LAL were prepared to investigate the effect of Zwittergent. Lots VII-82-0 and VII-82-Z were identical except that 0.02% Zwittergent was included in lot VII-82-Z. Lot VII-82-Z had a sensitivity to endotoxin of 1.6 pg/ml, while lot VII-82-0 was less sensitive (25 pg/ml). Lot VII-82-0 was almost as sensitive to lipid A as it was to endotoxin (50 pg/ml), but the LAL with Zwittergent was considerably less sensitive to lipid A (100 pg/ml). The results obtained with lots VII-82-0 and VII-82-Z suggested that the addition of Zwittergent increases sensitivity to endotoxin but interferes with the ability of the LAL reagent to react with both LRGs and lipid A.

To assess the role of Zwittergent in a formulated reagent which lacked only chloroform extraction, different concentrations of Zwittergent were added to the NOCHCL3 LAL reagent. This not only reduced the reactivity of the LAL to

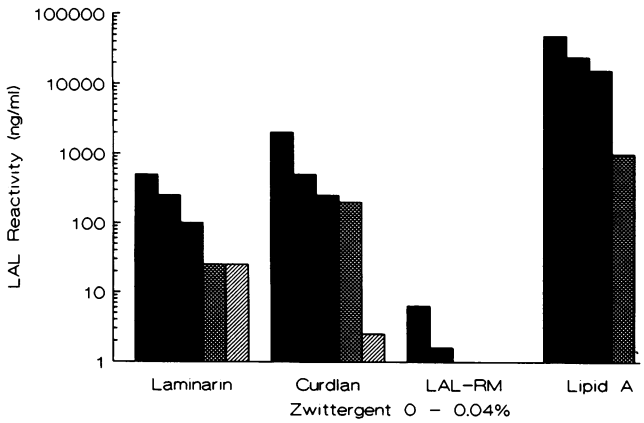

FIG. 1. LAL reactivity to laminarin, curdlan, LAL-RM, and lipid A by using the NOCHCL3 reagent with added Zwittergent concentrations of 0.0, 0.01, 0.02, 0.03, and 0.04% (bars from left to right, respectively). The concentrations of laminarin, curdlan, and lipid A were 1 mg/ml. The concentration of LAL-RM was not known, since it was an aqueous extract of a Cuprophan filter.

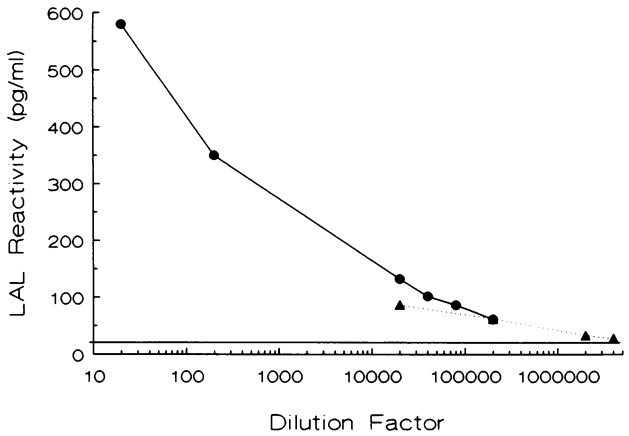

FIG. 2. Enhancement of the kinetic turbidimetric LAL reaction by curdlan. Increasing dilutions of curdlan were spiked with 25 pg of endotoxin per ml (line drawn at 25 pg/ml). The value of the reaction unspiked with curdlan was subtracted from the value of the reaction spiked with curdlan and was plotted against the dilution factor of curdlan. The initial concentration of curdlan was 1 mg/ml. Results of two experiments are shown.

LRGs but also reduced reactivity with lipid A (Fig. 1). These results represent the amount of activity in each sample as though it were due entirely to endotoxin. The dilution factor at the gel-clot endpoint was multiplied by the endotoxin sensitivity of the LAL reagent (25 pg/ml). The addition of 0.02 or 0.03% Zwittergent resulted in a substantial decrease in the reactivity of this LAL to LRGs and lipid A. These concentrations did not affect the sensitivity of this LAL reagent to standard endotoxin. The same inhibition of the reaction with LRGs was observed when Zwittergent was added to either Pyrotell or to a MAL-style formulation (Table 2). By using the MAL-style formulation, the sensitivity of the LAL reagent to endotoxin was increased with the addition of 0.02 and 0.03% Zwittergent, but it was dramatically decreased when 0.04% Zwittergent was added. There was no change in endotoxin sensitivity when Pyrotell was used. The reactivities of both reagents to laminarin were strongly inhibited.

Chloroform extraction of Zwittergent. Zwittergent is soluble in water and in chloroform. A 0.5% aqueous solution of Zwittergent was extracted with chloroform, and both the chloroform and aqueous layers were tested for sulfobetaine by spotting a thin-layer chromatography plate (Whatman International, Ltd., Maidstone, England). The plate was dried and developed in iodine vapors. An intense yellow spot from the chloroform layer clearly showed that Zwittergent can be extracted with chloroform.

Enhancement of the LAL test. When endotoxin is added to a sample and the amount measured by the LAL test is much greater than the amount added, the test result is deemed enhanced. Enhancement, as used in this study, can also be defined as an LAL reactivity greater than the sum of each component, i.e., endotoxin plus LRGs, when they are tested separately. By using the kinetic turbidimetric method and the LAL from ACC, enhancement of the LAL test was demonstrated with both curdlan and LAL-RM (Fig. 2 and 3, respectively). Dilutions were prepared, and each dilution was spiked with 25 pg of endotoxin per ml. The dilutions were assayed with and without the added endotoxin. In Fig.

TABLE 2. Effect of increasing Zwittergent concentrations on sensitivity to endotoxin and laminarin by using Pyrotell and MAL-style LAL

Zwittergent concn (%)	Sensitivity (pg/ml)[a]			
	MAL-style LAL		Pyrotell	
	Endotoxin	Laminarin	Endotoxin	Laminarin
0.0	6.25	2.5×10^3	6.25	$<8.0 \times 10^3$
0.01	3.125	1.6×10^4	6.25	$<8.0 \times 10^3$
0.02	1.56	1.0×10^6	6.25	1.6×10^4
0.03	1.56	2.5×10^6	6.25	5.0×10^6
0.04	25	5.0×10^7	ND[b]	ND

[a] Sensitivity is the minimum concentration of endotoxin or laminarin to give a positive gel-clot endpoint.
[b] ND, not done.

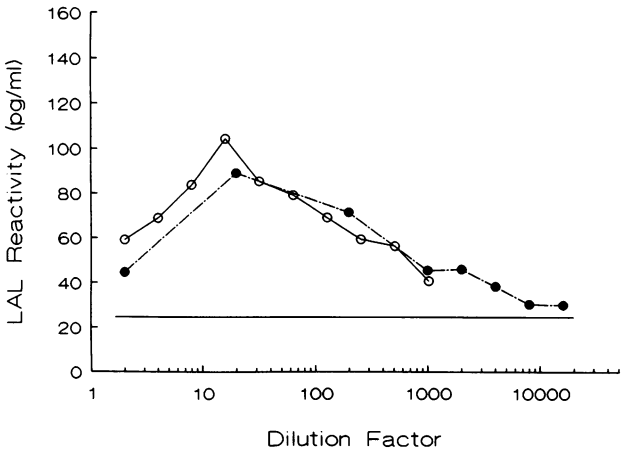

FIG. 3. Enhancement of the kinetic turbidimetric LAL reaction by LAL-RM. Increasing dilutions of LAL-RM were spiked with 25 pg of endotoxin per ml (line drawn at 25 pg/ml). The value of the reaction unspiked with LAL-RM was subtracted from the value of the reaction spiked with LAL-RM and was plotted against the dilution factor of LAL-RM. Results of two experiments are shown.

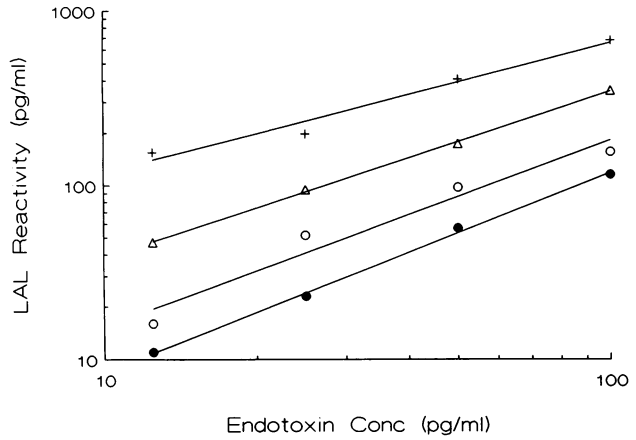

FIG. 4. Enhancement of standard endotoxin lines by three concentrations of curdlan: 250 ng/ml (+), 25 ng/ml (△), and 2.5 ng/ml (○). No curdlan was added to the standard line (●). Endotoxin standards were spiked with curdlan and were assayed by the kinetic turbidimetric method. The reactivities measured in the unspiked samples were subtracted from the reactivities measured in samples spiked with curdlan and were plotted against the endotoxin concentrations of the standard.

2 and 3, the endotoxin concentration of the sample was subtracted so that the curve depicts only enhancement. For example, in Fig. 2, when the activity of the 1:100,000 dilution or 10 ng of curdlan per ml alone (9.7 pg/ml) was subtracted from the activity of curdlan plus 25 pg of endotoxin per ml (106 pg/ml), there was a difference of 96.3 pg/ml, which is nearly fourfold more than the 25-pg/ml endotoxin spike. LAL-RM showed an increase in the amount of enhancement until a 1:10 dilution was reached, and then there was a gradual decrease. Curdlan enhanced the reaction greatly at high concentrations (25 μg/ml) and then gradually decreased. Laminarin demonstrated little or no enhancement of the kinetic turbidimetric LAL test (data not shown).

The enhancement of the kinetic turbidimetric test by curdlan was shown to be dependent on the curdlan concentration. Endotoxin standards were spiked with curdlan concentrations of 250, 25, and 2.5 ng/ml. Standard endotoxin curves were constructed with correlation coefficients of 0.98 and 0.99. The endotoxin measured in the unspiked sample was subtracted from that in the spiked sample, and the enhancement was plotted (Fig. 4). The enhancement was greatest when endotoxin was spiked with 250 ng/ml and was progressively less with 25 and 2.5 ng/ml.

Enzyme digestion experiments. Experiments were undertaken to determine whether the reactivity of glucan with LAL reagent could be reduced by enzyme digestion.

(i) Laminarinase. Laminarin is easily digested into glucose subunits by using laminarinase, with a concomitant six- to eightfold loss of reactivity to LAL (Table 3). The loss of reactivity was measurable, despite contamination of the enzyme with 20 ng of endotoxin per ml. When 1 mg of laminarin per ml was digested with 1 U of laminarinase per ml, 0.54 mg or more of glucose per ml was produced, providing assurance that most of the laminarin had been digested. This laminarinase had no effect on endotoxin. Laminarinase is a β-(1,3)-, β-(1,4)-hydrolase and may have digested some of the LAL-RM. Since the intrinsic endotoxin concentration of the enzyme was greater than that of LAL-RM, any small amount of enzymatic activity resulting in a decrease in LAL activity would be masked.

TABLE 3. Digestion of glucans with laminarinase and cellulase

Substrate	Reactivity (ng/ml)[a]			
	Laminarinase		Cellulase	
	No enzyme	Digested	No enzyme	Digested
Laminarin	4,000	62.5	8,000	2,000
Curdlan	1,000	31.25	ND[b]	ND
LAL-RM	10	10	10	0.125
Water	0	20	0	0.025
Endotoxin	0.1	20	0.1	0.062

[a] LAL reactivity was measured before and after enzyme digestion. Reactivity is the dilution factor at the gel-clot endpoint × sensitivity of the LAL reagent.
[b] ND, not done.

(ii) Cellulase. By using the gel-clot method and Pyrotell, the activity of LAL-RM was reduced from 10 to 0.125 ng/ml after digestion with cellulase (Table 3). The amount of activity left could be attributed to the intrinsic endotoxin contamination of the cellulase, since water plus enzyme had activity nearly identical to that of the digested LAL-RM. Cellulase had no effect on endotoxin. This enzyme reduced the activity of laminarin from 8,000 to 2,000 ng/ml and, therefore, is most likely contaminated with a trace amount of a β-(1,3)-hydrolase.

The digestion experiments support the contention that LAL-RM is a glucan with primarily β-(1,4) linkages and that it is different from laminarin, which has predominantly β-(1,3) linkages.

DISCUSSION

There is little doubt that LAL, both raw and formulated, reacts with laminarin, curdlan, and LAL-RM. The amount of reactivity is low, at least 1,000-fold less than the reactivity to endotoxin. Since raw, unextracted LAL with no additives reacts with LRGs, it is clear that this is an inherent property of the *Limulus* amebocyte. It could be argued that reactivity with β-(1,3)-glucans is another defensive strategy of *Limulus* against yeast or fungus contamination, just as the endotoxin-induced *Limulus* clotting reaction is regarded as a defense mechanism against gram-negative bacterial infection.

These results substantially agree with those of Söderhall et al. (28), who reported reactivity with curdlan but not with laminarin when a LAL reagent with no chloroform extraction and no added Zwittergent was used. In the present study, there was reactivity with curdlan and minimal reactivity with laminarin when those LAL reagents with no chloroform extraction were used.

There is no reliable way to know how much, if any, endotoxin was present in the glucan preparations used in this study, since the LAL test is the most sensitive test available for the detection of endotoxin. However, analysis of laminarin for the hydroxy fatty acids specific to endotoxin, using positive ion chemical ionization-mass spectroscopy, detected the equivalent of 3.7 ng of LPS per ml (30a). This value was 10-fold less than the amount of LAL reactivity left after treatment with laminarinase (62.5 ng/mg). Curdlan was routinely dissolved and stored in 0.1 N NaOH. This concentration of NaOH is often used for depyrogenation of glassware, filters, and chromatographic columns and is more effective at 37°C. Control experiments have shown that 0.1 N NaOH inactivates 90% of a 1,000-pg/ml solution of endotoxin in 2 h at room temperature. Laminarin (1 mg/ml)

treated with 0.1 N NaOH for 4 h at 37°C lost approximately 25% of its LAL reactivity. Curdlan heated at 37°C for 2 h in the presence of NaOH lost no reactivity (unpublished data). Thus, we assume that curdlan contained little endotoxin, while the laminarin may have contained as much as 20 to 60 ng/mg.

The contamination of these glucan preparations with endotoxin is a matter for concern, particularly since it has been shown that glucans can enhance the LAL-endotoxin reaction and that the enhancement is glucan dependent. Thus, some but not all of the LAL reactivity seen may be attributable to endotoxin or may be an enhanced reaction.

The conformation and degree of polymerization of the glucan molecule represents a potential source of variability for LAL reactivity. The reactivity of the LAL reagent Toxicolor (Seikagaku Kogyo Co., Tokyo, Japan) was related to the molecular weight, the degree of branching, and the conformation of the glucan. The β-(1,3)-glucans with higher molecular weights were more active than those with lower molecular weights, and single-stranded glucans were more active than those with a helical structure (20).

Polysaccharides, other than laminarin or curdlan, have been tested for LAL reactivity by others. These include simple dextrans and synthetic dextran derivatives (15, 30). One investigator suggested that lipid A might not be wholly responsible for initiating the LAL coagulation cascade, but that the polysaccharide moiety of the LPS molecule may play a small role (15). The O antigen of LPS consists of repeating oligosaccharides. The O antigen of the *E. coli* LPS contains a glucan core oligosaccharide made up of α-(1,2) and α-(1,3) linkages and an O antigen-specific polysaccharide of mixed hexoses linked with various α and β configurations (11). Most O antigens from other gram-negative bacteria, although differing in the individual monosaccharides which make up the repeating oligosaccharides, share a similar general structure.

The loss of activity of LAL-RM following digestion with cellulase agrees with an earlier report from Pearson et al. (24) and provides evidence that LAL-RM is a glucose polymer with substantial β-(1,4) linkages. In contrast, Ohno et al. (20) concluded that only glucans with a β-(1,3) linkage are reactive with LAL. While their study examined glucans with both β-(1,3) and β-(1,4) linkages, LAL-RM from Cuprophan filters was not included.

Factor G, a protein component of LAL as well as *Tachypleus* amebocyte lysate, has been proposed as an enzyme which is activated by LRGs. Activated factor G, in turn, activates the pro-clotting enzyme (12, 16). Chromatography of *Limulus* lysate yields a peak which contains both factor G and the natural substrate coagulogen. One LAL reagent, Endospecy (Seikagaku Kogyo Co. Ltd.), has been produced; this reagent does not contain this peak and, when it is combined with a chromogenic substrate to replace coagulogen, reacts with endotoxin but not with glucans (19).

In this study, the different reactivities of the LAL reagents to LRGs and lipid A were shown to be due to different formulations. The addition of Zwittergent increased the sensitivity of an experimental formulation (lot VII-82-Z) to endotoxin but had little effect on the endotoxin sensitivity of either the NOCHCL3 or the Pyrotell reagents. The sensitivity of all LAL reagents tested was almost the same with lipid A and endotoxin except those of MAL and WB, and it was shown that increasing Zwittergent concentrations decreased the reactivity to lipid A. In all LAL reagents tested, there was decreased reactivity to LRGs when Zwittergent was present.

The effect of Zwittergent is not understood. Although Zwittergent is not generally thought to interfere with the biological activities of a number of membrane proteins, at least one report exists describing selective enzyme inhibition by sulfobetaines (21). It is possible, therefore, that the sulfobetaine Zwittergent 3-14, at concentrations of ≥0.02%, selectively inhibits factor G or other factors in LAL which react with LRGs.

The action of Zwittergent on LPS may consist of a conformational alteration, making LPS more or less available to the LAL reagent. LPS exists as an aggregate and often in a micellar structure, and Zwittergent may cause disaggregation of LPS or endotoxin and may increase its reactivity with LAL. Other chemicals, such as triethylamine, which forms an LPS salt, have been shown to increase the sensitivity of LAL to endotoxin (7, 26). Zwittergent increases LAL sensitivity up to a concentration of about 0.03%, beyond which sensitivity decreases. If Zwittergent affected the solubility of both LPS and lipid A, one would not expect equal changes in LAL reactivity, since the initial solubilities of these compounds are quite different. From our results, it appears that lipid A is more sensitive than LPS to the effects of Zwittergent.

Chloroform extraction of LAL increased the sensitivity of the reagent not only to endotoxin and lipid A but also to LRGs. LAL-RM reacted with both the experimental LAL reagent NOCHCL3, which had no chloroform extraction, and Pyrotell. Reactivity was lower with the NOCHCL3 LAL reagent. Therefore, the reactivity of LAL-RM from Cuprophan filters was increased, but it was not caused by chloroform extraction. These results seem to contradict the conclusion of Pearson et al. (23), who reported as unpublished data an experiment in which Pyrogent was extracted with chloroform and became reactive with LAL-RM. However, since Zwittergent is extracted from aqueous solution by chloroform, the results of Pearson et al. (23) are in agreement with our data, but they are better explained as the removal of the inhibitor Zwittergent.

The precise mechanism whereby chloroform increases LAL sensitivity to both endotoxin and LRGs is not known. Chloroform extraction removes lipids and lipoproteins from solution and also denatures some proteins, any one of which could be an inhibitor (29).

Some of the biological activities ascribed to glucans, such as tumor necrosis, macrophage activation, and activation of complement, are also caused by endotoxin. There is some question whether a small amount of endotoxin contamination in a glucan can cause the biological activity. However, enough controlled studies exist to give credence to the effects that can be attributed solely to glucans and to a recognition that LRGs are potential health hazards.

Since glucan reactivity can compromise the endotoxin specificity of LAL, a necessary adjunct to the LAL test is a simple test which differentiates between glucan and endotoxin. While LRGs would be considered a contaminant if they were present in pharmaceutical products, a positive LAL test would indicate a problem regardless of the nature of the contaminant. However, a glucan (or endotoxin)-specific test would help the pharmaceutical manufacturer locate the source of contamination or would help the clinician differentiate gram-negative bacteremia from candidiasis or other fungemia. A glucan-specific test, the G test, has been reported, but it is not commercially available (19). An endotoxin-specific LAL reagent, Endospecy, is available in Japan, but it is not licensed for pharmaceutical or diagnostic use in the United States. As reported here and by others

(15), a simple, qualitative test for differentiating LRGs from endotoxin consists of treating the sample with 0.1 N NaOH. Samples of LRGs will not lose LAL reactivity in the presence of 0.1 N NaOH, while those with endotoxin will have diminished reactivity because of mild base hydrolysis.

In conclusion, we showed that LAL reactivity to glucans is dependent on the nature of the LAL reagent preparation. While chloroform-extracted LAL shows increased sensitivity to LPS (including lipid A) and LRGs, LAL formulated with Zwittergent exhibits decreased sensitivity toward lipid A and LRGs, with slightly increased sensitivity to LPS. Since certain glucans have been shown to be biologically active in vivo, a LAL reagent (without Zwittergent) may serve a dual purpose of indicating endotoxin or glucan contamination of parenteral drugs or medical devices.

ACKNOWLEDGMENTS

We thank Michael E. Dawson, Marilyn J. Gould, Norman R. Wainwright, Alfred F. Borg, and Robert, J. Miller for many helpful suggestions and assistance.

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