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Differential Blocking of Coagulation-Activating Pathways of *Limulus* Amebocyte Lysate

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The coagulation of *Limulus* amebocyte lysate (LAL) can be activated through two pathways, one initiated by endotoxin and the other by β-glucans. The two pathways join at the step of activation of the proclotting enzyme. We report here that the endotoxin-activated pathway can be differentially inhibited by two methods in a *Limulus* enzyme-linked immunosorbent assay (ELISA), either by the combined use of dimethyl sulfoxide and polymyxin B or by a monoclonal antibody against *Limulus* factor C. LAL reactivities to 10 different endotoxin preparations could be inhibited by the former method by a factor of 10^4 to 10^6 and could be blocked almost totally by the latter method, irrespective of the source of endotoxin. The sensitivity of the assay was approximately 50 pg/ml both for curdlan from *Alcaligenes faecalis* and for laminarin from *Laminaria digitata*. We also found that the β-glucan-activated pathway could be totally blocked by laminarin (>1 μg/ml) without affecting the endotoxin-activated pathway, allowing endotoxin to be quantitated specifically by the *Limulus* ELISA with a detection limit of 0.005 endotoxin unit per ml. The use of uninhibited and differentially inhibited ELISAs demonstrated that different LAL preparations showed much greater variation in assaying β-glucans than in assaying endotoxins. The LAL reactivity of normal human plasma was found to be due to the activation of the β-glucan pathway, but not the endotoxin pathway, of LAL.

Limulus amebocyte lysate (LAL) is known to contain a coagulation system that can be activated by a minute amount of bacterial endotoxin (lipopolysaccharide [LPS]). The coagulation cascade consists of three proenzymes (factor C, factor B, and proclotting enzyme) and one clottable protein (coagulogen) (7, 14). LPS activates factor C, which then activates factor B, which in turn converts proclotting enzyme to clotting enzyme. Clotting enzyme cleaves coagulogen to yield an insoluble coagulin gel. The formation of gel clot and measurement of clotting enzyme activity by means of a synthetic chromogenic substrate constitute, respectively, the bases of the gel clot and chromogenic LAL assays for the detection of endotoxins (8, 15). We have recently developed sensitive endotoxin assays based on the immunochemical detection of *Limulus* coagulogen or its cleaved fragment, peptide C, with detection limits as low as 0.001 endotoxin unit (EU) per ml (2, 24, 25).

Because of its extreme sensitivity to endotoxins, the LAL test has been considered a promising means of detecting endotoxin in clinical settings (9). However, its specificity for endotoxin appears to be compromised by certain β-glucans, originating from fungi (5, 6, 10) or hemodialysis membranes (3, 23), which are also LAL reactive, although these glucans are at least 1,000-fold less active than endotoxin. Fortunately, the activation of LAL by β-glucan occurs through an initiating factor different from that for endotoxin, namely, factor G (16). Separation of the two pathways by fractionation and reconstitution of the relevant factors has been reported recently (12, 19). Addition of curdlan, a carboxymethylated β-glucan, to LAL at high concentrations (>1 mg/ml) has also been shown to block the β-glucan pathway (11). Another study shows that the surfactant Zwittergent interferes with LAL reactivity to β-glucan (20). However, the reconstituted LAL fractions are not stable, Zwittergent also affects lipid A to some extent, and curdlan tends to autopolymerize to form an insoluble gel at high concentrations after storage for 3 months, even at 4°C (unpublished observation).

In this study, we have attempted to develop specific LAL tests for endotoxin and β-glucan by differentially blocking the two activation pathways of LAL, using our newly developed *Limulus* peptide C enzyme-linked immunosorbent assay (ELISA) to detect reactivity. We have also assessed the influence of different formulations of LAL on the quantification of endotoxin or β-glucan activities of various laboratory specimens.

MATERIALS AND METHODS

All glassware was rendered pyrogen free by being heated to 250°C for at least 3 h. Sterile, pyrogen-free tips and microplates were purchased from Eppendorf, Hamburg, Germany, and Nunc, Roskilde, Denmark, respectively. The LAL reagent water used was confirmed to have less than 0.001 EU/ml by the LAL test.

LAL preparations. Commercial LAL preparations included Pyrotell (lot 42-99-541) from Associates of Cape Cod (ACC), Woods Hole, Mass., and LAL (lot 2L0860) from Whittaker Bioproducts (WB), Walkersville, Md. The *Tachypleus* amebocyte lysate (TAL) was prepared from the horseshoe crab *Tachypleus tridentatus* without chloroform extraction, as described previously (25).

LPS. The control standard endotoxin was NP-3, which is a purified preparation of LPS from *Salmonella abortus equi* purchased from Pyroquant Diagnostik, Walldorf, Germany, and its potency was confirmed to be 10 EU/ng in comparison with USP reference standard endotoxin EC-5. The following LPS preparations, obtained by phenol extraction, were purchased from Sigma Chemical Co., St. Louis, Mo.: *Escherichia*

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coli O111:B4 (determined potency, 2.5 EU/ng), *E. coli* O55:B5, *E. coli* O127:B8, *E. coli* O128:B12, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *S. abortus equi*, *Salmonella enteritidis*, *Salmonella minnesota*, *Salmonella typhimurium*, and *Shigella flexneri*.

Glucan preparations. Laminarin isolated from *Laminaria digitata* was obtained from Sigma. Highly purified curdlan from *Alcaligenes faecalis* was purchased from Wako Pure Chemical Industries, Osaka, Japan. Both laminarin and curdlan were dissolved in 0.2 N NaOH at 5 mg/ml and incubated at 56°C for 6 h to inactivate contaminating endotoxin (25).

Polymyxin B. Polymyxin B sulfate was purchased from Calbiochem, La Jolla, Calif. The endotoxin and β-glucan-like activities found in polymyxin B were removed by adsorption with activated charcoal (Merck, Darmstadt, Germany); 0.1 g was mixed with 10 ml of polymyxin B solution (20 mg/ml) at 37°C for 1 h, and this was followed by centrifugation to remove the charcoal.

MAb against factor C. Partially purified factor C was obtained from TAL by dextran sulfate–Sepharose CL-6B chromatography as described by Nakamura et al. (17) and was further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) carried out with a 12% gel, essentially as described by Laemmli (13). The band at 120 kDa was cut out and eluted with an electroelutor (Bio-Rad Laboratories, Richmond, Calif.). Purified factor C was adsorbed to aluminium hydroxide suspension (2 mg/ml in normal saline) and injected intraperitoneally (0.5 ml, containing 25 μg of factor C, per mouse) into five female CF1 × BALB/c mice. Booster doses were given at 2-week intervals, and animals were test bled 10 days after each boost. When high antibody titers were found by ELISA in factor C-coated microtiter plates, spleen cells were fused by a standard procedure (4) with myeloma cells from line X63/Ag8.6.5.3. Selected monoclonal antibodies (MAbs) against factor C were purified from hybridoma supernatants by affinity chromatography on protein A–Sepharose CL-4B columns (Pharmacia, Uppsala, Sweden). One of the MAbs (MAb-B2) was found to react with both *Tachypleus* factor C and *Limulus* factor C, since it recognized the 120-kDa band of the partially purified factor C preparations from both LAL and TAL in SDS-PAGE immunoblotting (unpublished observation), and was chosen for use in the present study. The endotoxin contamination of preparations containing 1 mg of MAb-B2 per ml was about 100 ng/ml and was removed by Triton X-114 extraction (1).

Preparation and pretreatment of plasma. Blood from human donors was drawn into pyrogen-free glass tubes containing pyrogen-free heparin (final concentration, 4 IU/ml). Plasma was separated by centrifugation at 500 × *g* for 15 min. Perchloric acid (PCA) precipitation of plasma to remove factors that interfere with LAL was carried out essentially as described by Obayashi (18) but with one minor modification: the neutralized supernatant was further diluted twofold in the LAL reagent water.

***Limulus* peptide C ELISA.** Unless otherwise stated, the *Limulus* peptide C ELISA was performed as previously described (25). When MAb-B2 was used as a blocking agent, stock solution (1 mg/ml) was added to diluted LAL and incubated at 4°C for 20 min before the ELISA was performed. When dimethyl sulfoxide (DMSO) and polymyxin B were used as blocking agents, the ordinary LAL buffer was replaced by buffer containing 20% (vol/vol) DMSO, 100 μg of polymyxin B per ml, 60 mM $MgCl_2$, and 100 mM Tris-HCl, pH 8.

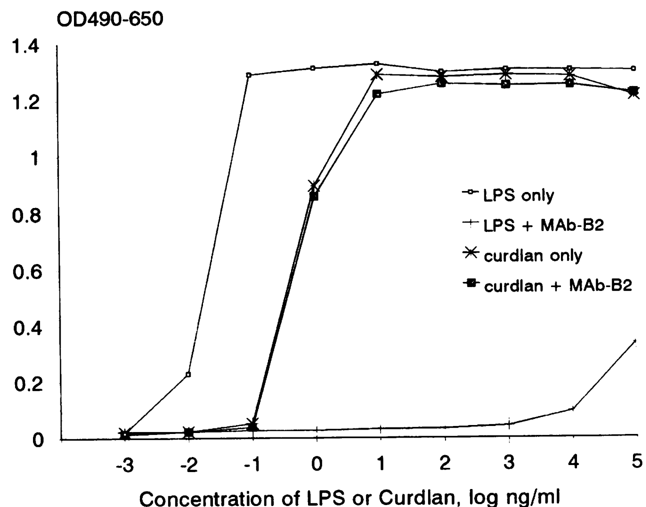

FIG. 1. Differential inhibition of the LPS-activated pathway of LAL by MAb-B2 (10 μg/ml). OD490-650, optical density at 490 nm corrected by optical density at 650 nm.

RESULTS

Differential blocking of the endotoxin-activated LAL pathway. MAb-B2 is a murine MAb raised against factor C from TAL and was found to cross-react with factor C from LAL (see Materials and Methods). Figure 1 shows the effect of MAb-B2 on the reactivity of LAL to LPS (from *E. coli* O111:B4) and to curdlan. LAL (from ACC) became almost unreactive to LPS in the presence of MAb-B2 at 10 μg/ml, while the reactivity of LAL to curdlan was unaltered. The same effect was also observed with other LPS preparations, as shown in Table 1.

Figure 2 shows the dose-dependent inhibition of the endotoxin-activated pathway of LAL by MAb-B2. When the concentration of the antibody in LAL was at or above 10 μg/ml, the reactivity of LAL to LPS was almost totally inhibited. No further inhibition was observed when the MAb-B2 concentration was increased above 20 μg/ml. The residual LAL reactivity of LPS at concentrations of >100 μg/ml was presumed to be due to the contamination of the LPS preparations with β-glucan-like substances, since it could not be abolished even by treatment with 0.2 N NaOH at 60°C for 24 h.

TABLE 1. Inhibition of the LAL-LPS reaction by DMSO plus polymyxin B (D-P) or by MAb-B2

Source of LPS	Detection limit (ng/ml) with:		
	LAL only	LAL + D-P[a]	LAL + MAb-B2[b]
E. coli O55:B5	0.001	100	>10,000
E. coli O127:B8	0.001	2,500	>10,000
E. coli O128:B12	0.0005	1,000	>10,000
K. pneumoniae	0.001	1,250	>10,000
P. aeruginosa	0.001	250	>10,000
S. abortus equi	0.002	20	>10,000
S. enteritidis	0.002	250	>10,000
S. minnesota	0.002	100	>10,000
S. typhimurium	0.003	1,000	>10,000
Shigella flexneri	0.005	2,500	>10,000

[a] The LAL buffer containing 20% DMSO, 100 μg of polymyxin B per ml, 60 mM $MgCl_2$, and 100 mM Tris-HCl (pH 8) was used to dilute LAL in the *Limulus* peptide C ELISA.

[b] The concentration of MAb-B2 in LAL was 10 μg/ml.

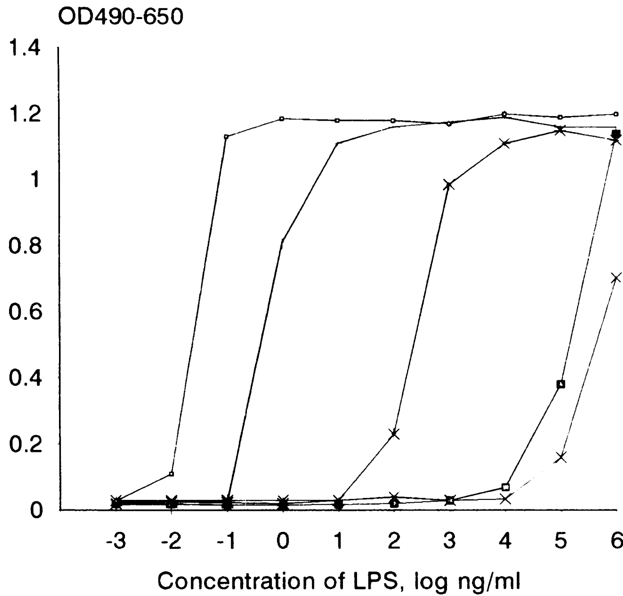

FIG. 2. Dose-dependent inhibition of the LPS-activated pathway of LAL by MAb-B2 at 0 (□), 2.5 (+), 5 (*), 10 (■), and 20 (×) μg/ml. OD490-650, optical density at 490 nm corrected by optical density at 650 nm.

Figure 3 shows the effect of the combined use of 20% DMSO and polymyxin B (100 μg/ml) on the reactivity of LAL to LPS (from *E. coli* O111:B4). The reaction of LAL with LPS was inhibited by a factor of more than 10^5 (increasing the detection limit from 2.5 pg/ml to 1 μg/ml), but the reactivity of LAL to curdlan was not significantly altered. The inhibitory effect of DMSO and polymyxin B on the reactivity of LAL to other LPS preparations was variable, ranging from a factor of 10^4 to 10^6, as shown in Table 1.

Differential blocking of the β-glucan-activated LAL pathway. Figure 4 shows the reaction curves of LAL with LPS, laminarin, and curdlan and the effects of laminarin and curdlan on the β-glucan-activated pathway. The concentration ranges in which laminarin and curdlan reacted with LAL were from 0.05 ng/ml to 1 μg/ml and from 0.1 ng/ml to 500 μg/ml, respectively. Addition of laminarin at 5 μg/ml or curdlan at 1 mg/ml to LAL totally blocked the β-glucan-activated pathway without compromising the reactivity of LAL to LPS.

The reactions of TAL with LPS and curdlan were found to be similar to those of LAL, but TAL did not react with laminarin at any concentration from 1 pg/ml to 1 mg/ml (25). Addition of laminarin to TAL at a concentration as low as 0.1

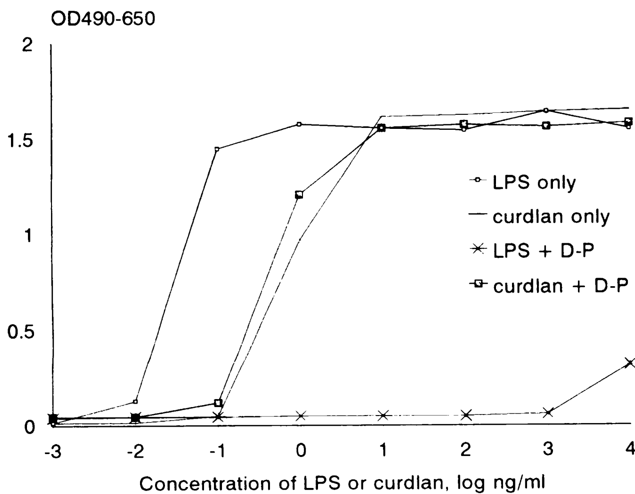

FIG. 3. Differential inhibition of the LPS-activated pathway of LAL by the combined use of 20% DMSO and 100 μg of polymyxin B per ml (D-P). OD490-650, optical density at 490 nm corrected by optical density at 650 nm.

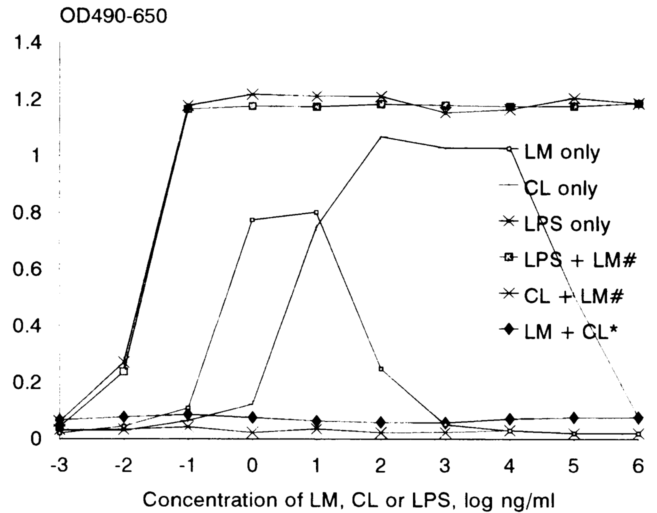

FIG. 4. Differential inhibition of the β-glucan-activated pathway of LAL by laminarin (LM) or curdlan (CL). #, Concentration of LM in LAL was 5 μg/ml; *, concentration of CL in LAL was 1 mg/ml. OD490-650, optical density at 490 nm corrected by optical density at 650 nm.

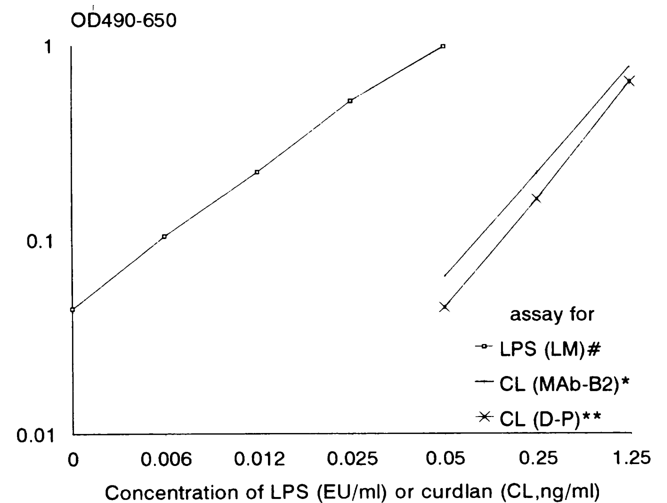

FIG. 5. Standard curves of specific *Limulus* peptide C ELISAs for LPS or curdlan. #, the concentration of laminarin (LM) in LAL was 5 μg/ml. *, the concentration of MAb-B2 in LAL was 10 μg/ml. **, the LAL buffer containing 20% DMSO, 100 μg of polymyxin B per ml, 60 mM $MgCl_2$, and 100 mM Tris-HCl (pH 8) was used to dilute LAL in the *Limulus* peptide C ELISA. OD490-650, optical density at 490 nm corrected by optical density at 650 nm.

TABLE 2. Determination of LPS and β-glucan by using different formulations of LAL and TAL

LAL or TAL formulation	Content of LPS or β-glucan (ng/ml)[a] in specimen no.[b]					
	1	2	3	4	5	6
LPS and β-glucan reactive						
LAL (ACC)	29.8	25.7	25.1	21.4	2.43	0.024
LAL (WB)	22.9	29.8	33.4	58.0	5.13	0.038
TAL	15.5	13.7	13.5	10.7	0.99	<0.005
CV (%)[c]	31.5	36.3	41.6	82.6	73.3	
LPS reactive						
LAL (ACC) + LM[d]	13.2	7.5	10.1	<0.1	<0.1	<0.005
LAL (WB) + LM	16.2	6.4	8.4	<0.1	<0.1	<0.005
TAL + LM	12.6	4.4	6.7	<0.1	<0.1	<0.005
CV (%)	13.8	25.7	20.2			
β-Glucan reactive						
LAL (ACC) + MAb-B2[e]	98.5	1,566	1,003	1,825	250	2.42
LAL (ACC) + D-P[f]	90.6	1,740	960	1,204	186	1.82
CV (%)	5.9	7.4	3.1	28.9	20.6	20.0

[a] Standards for LPS and β-glucan were NP-3 and curdlan, respectively. The data are mean values of triplicate determinations. For the formulations reactive with both LPS and β-glucan, values are LPS equivalents.

[b] Specimens 1, 2, and 3 were naturally contaminated human immunoglobulin preparations. Specimen 4 was a culture medium (brain heart infusion). Specimen 5 was a culture supernatant of *Candida albicans*. Specimen 6 was a pool of normal human plasma (from six donors) pretreated by the PCA method.

[c] Coefficient of variation of the results obtained with different formulations of LAL and TAL.

[d] The concentration of laminarin (LM) in LAL was 5 μg/ml.

[e] The concentration of MAb-B2 in LAL was 10 μg/ml.

[f] D-P, DMSO-polymyxin B. The LAL buffer containing 20% DMSO, 100 μg of polymyxin B 100 per ml, 60 mM $MgCl_2$, and 100 mM Tris-HCl (pH 8) was used to dilute the LAL in the *Limulus* peptide C ELISA.

μg/ml was sufficient to block the β-glucan pathway activated by curdlan.

Standard curves of *Limulus* peptide C ELISAs specific for LPS or β-glucan. Figure 5 shows a linear relationship (on a double logarithmic plot) of the LAL reaction with LPS when laminarin (5 μg/ml) was added to LAL to block the β-glucan-activated pathway and also shows a linear relationship of the reaction with β-glucan when either MAb-B2 or DMSO and polymyxin B were added to block the LPS-activated pathway. The detection limit for LPS in the LPS-specific ELISA was 0.005 EU/ml, while the detection limit for curdlan or laminarin in the β-glucan-specific ELISA was about 50 pg/ml. Curdlan was found to be unstable in solution at concentrations of >1 mg/ml, since it autopolymerized to form an insoluble gel after storage at 4°C for 3 months. Laminarin was observed to be stable at 1 mg/ml in 50 mM NaOH at 4°C for at least 1 year.

Comparison of different formulations of LAL and TAL for the determination of LPS and β-glucan in specimens. As shown in Table 2, the conventional LAL or TAL preparations, which react with both LPS and β-glucan, gave much more variable results (the coefficient of variation was more than double) for specimens that contained primarily β-glucans (samples 4 and 5) than for specimens that contained both LPS and β-glucans (samples 1, 2, and 3). The LAL or TAL made specific for LPS by the addition of laminarin (5 μg/ml) reacted little with the specimens containing primarily β-glucans and produced less variation than the conventional preparations. The LAL made specific for β-glucan by the addition of MAb-B2 gave results comparable to those obtained by the addition of DMSO and polymyxin B. PCA-treated normal human plasma exhibited some β-glucan-like activity, but no endotoxin activity, when LAL preparations were used and diluted fourfold.

DISCUSSION

The LAL test is widely used as a sensitive assay for endotoxin. It is now known that the coagulation of LAL can be induced not only by LPS but also by certain β-glucans, which act through different initiating factors. LPS activates factor C and β-glucan activates factor G, although the latter has not been fully characterized (14). MAb-B2, used in this study, is a MAb raised against *Tachypleus* factor C but is found to react with and inhibit both *Limulus* factor C and *Tachypleus* factor C. This indicates the close similarity of the factors C from these species, which have also been demonstrated to have the same molecular size and functions (22). While the exact mechanism of MAb-B2 inhibition of the activation of factor C by LPS is not clear, it is possible that the binding of factor C to MAb-B2 prevents its binding to LPS and consequent activation through an allosteric effect or one of steric hindrance by MAb-B2, as suggested by the fact that premixing of the antibody with LAL or TAL at 4°C for about 15 to 30 min is required to ensure effective blocking of the LPS-activated pathway.

The use of laminarin to block the β-glucan-activated pathway has advantages over the use of curdlan in that laminarin is stable in solution and is effective at a concentration at least 100-fold lower than that of curdlan. Commercial curdlan and laminarin, in solutions of 5 mg/ml, contain about 1 ng of endotoxin per ml. The contaminating endotoxin can be totally destroyed by heating to 50 to 60°C in 0.2 N NaOH for about 6 h, without affecting the LAL reactivities of curdlan and laminarin. However, the curdlan solutions subjected to this treatment seem to be more susceptible to gelling, probably because of autopolymerization, than untreated solutions (gelling within 3 months after treatment versus 1 year). It is desirable to use as differential blocking agents those β-glucans that have no reactivity with LAL at any concentration and that can produce total inhibition of the β-glucan-activated pathway without affecting the LPS-activated pathway. Tanaka et al. (21) have recently reported the inhibition of high-molecular-weight (1-3)-β-D-glucan-dependent activation of *Limulus* coagulation factor G by laminaran oligosaccharides and curdlan degradation products.

Inhibition of the LPS-activated pathway by the combined use of DMSO and polymyxin B is based on the LPS-neutralizing effect of polymyxin B and the inhibitory effect of DMSO on factor C (unpublished observation). The inhibition of the LAL reactivity of LPS by polymyxin B alone at 100 μg/ml or by 20% DMSO alone is limited, increasing the detection limit of the assay to about 10 ng of LPS per ml, but combining the two reagents produces a synergistic effect.

Greater variations occur among different LAL and TAL preparations in quantifying β-glucans than in quantifying LPS, and this probably stems from the different reactivities of these preparations to curdlan, which is used as a control standard for β-glucan, and to other naturally occurring β-glucans. For example, the TAL prepared in our laboratory and the LAL from WB used in this study are amebocyte lysates that have not been subjected to chloroform treatment, and these preparations have been found to react with curdlan but not with laminarin, while the LAL from ACC, which is a chloroform-treated preparation, reacts with curdlan and laminarin with almost equal sensitivities (25). The great variation of different LAL and TAL preparations in their reactivities with β-glucans

may complicate the standardization of a *Limulus* β-glucan assay.

Normal human plasma pretreated with PCA exhibits some β-glucan-like activity when the LAL is diluted more than twofold but shows no endotoxin activity regardless of the dilution of the LAL. The greater the dilution of LAL used, the more the β-glucan activity is observed. A similar phenomenon has been observed when normal human plasma is pretreated by the diluting-heating method (the plasma is diluted 10-fold in water and heated to 75°C for 5 min). Whether the observed β-glucan-like activity is a genuine property of normal plasma or is caused by denaturation of plasma constituents during PCA or heat treatment remains to be investigated.

In conclusion, this study has established specific *Limulus* peptide C ELISA methods for determination of either endotoxin or β-glucan by differentially blocking the coagulation-activating pathways of LAL. The blocking agents developed in this study may in principle be applied to other quantitative LAL assays, such as the chromogenic and turbidimetric assays, although their effective concentrations may well be different in view of the fact that the LAL is diluted at least fourfold in the *Limulus* peptide C ELISA. Optimization studies and assessment of the clinical applications of these assays for the diagnosis of endotoxemia and fungemia are being carried out.

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