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Sensitive Quantitation of Endotoxin by Enzyme-Linked Immunosorbent Assay with Monoclonal Antibody against *Limulus* Peptide C

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***Limulus* peptide C, a 28-amino-acid fragment of coagulogen formed by the reaction of endotoxin with *Limulus* amebocyte lysate, was synthesized, and a monoclonal antibody against it was raised. A new microassay for endotoxin was developed, using this antibody in an enzyme-linked immunosorbent assay for generated peptide C-like immunoreactivity. A linear relationship between absorbance and endotoxin concentration was obtained. Control standard endotoxin in water could be detected to a level of 0.001 endotoxin unit per ml. The endotoxin levels in plasma samples from normal humans, rabbits, mice, and guinea pigs were generally found to be below the detection limit of 0.01 endotoxin unit per ml of plasma. The color and turbidity of specimens did not interfere with the assay. The consumption of *Limulus* amebocyte lysate in the assay was less than 5% of that in the gel-clot and chromogenic assays. With raw lysate, which was much more stable in solution than chloroform-treated lysate, the assay was still highly sensitive to endotoxin but was totally unresponsive to natural glucans. The monoclonal antibody cross-reacted with peptide C-like immunoreactivity generated in *Tachypleus* amebocyte lysate, which gave equal sensitivity in the endotoxin assay.**

The *Limulus* amebocyte lysate (LAL) test is by far the most sensitive assay for bacterial endotoxins. LAL is prepared from circulating *Limulus* (horseshoe crab) amebocytes and contains a coagulation system that may be considered a prototype of mammalian blood coagulation, which involves the sequential activation of several proenzymes (7, 11). Endotoxin is known to activate the initial enzyme (factor C) of the LAL coagulation system, ultimately leading to the conversion of coagulogen, a clottable protein, into coagulin and peptide C. Visible formation of a coagulin gel-clot generally indicates activation of the LAL by endotoxin and constitutes the basis of the gel-clot method for endotoxin detection. The gel-clot assay is simple to perform but lacks an objective end point and is not strictly quantitative. Later developments include turbidimetric and chromogenic LAL assays (8, 24); both are quantitative, objective, and more sensitive. Kinetic versions of these assays have recently been developed (12, 19), but they are unsuitable for the colored or turbid specimens that are often encountered in clinical and laboratory use.

The LAL test was originally considered to be specific for endotoxins (11). However, some β-glucans and β-glucan-containing mycotic products have subsequently been found to be LAL reactive (1, 2, 4–6, 9). These glucans include curdlan, laminarin, and LAL-reactive materials (2, 16, 17). A recent study indicates that the reactivity of LAL with β-glucan is greatly influenced by the formulation of the LAL reagent (20). The initiation of LAL coagulation by endotoxin has been shown to be independent of that by β-glucan (13).

We have previously developed an endotoxin assay by combining the use of LAL with an enzyme-linked immunosorbent assay (ELISA) for coagulogen (25). This endotoxin assay is sensitive and well suited for the determination of endotoxins in plasma samples because it is not subject to interference from the color or turbidity of the specimens, but it takes a relatively long time to perform (usually 5 to 6 h). In the present study, we have synthesized *Limulus* peptide C, a peptide fragment of coagulogen, and developed an ELISA with a monoclonal antibody (MAb) raised against this peptide. We have found that the generation of peptide C-like immunoreactivity in the course of the LAL-endotoxin reaction could be detected much more readily than the conversion of coagulogen and correlated well with endotoxin concentrations. Using this assay, we have also tested the reactivities of various LAL preparations to β-glucans. We have confirmed the functional identity of LAL with *Tachypleus* amebocyte lysate (TAL) for the determination of endotoxin by means of this assay.

MATERIALS AND METHODS

All glassware was rendered pyrogen free by heating 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. LAL reagent water was confirmed to have less than 0.001 endotoxin unit (EU) per ml by the LAL test.

LAL preparations. Commercial LAL preparations included Pyrotell (lot 42-99-541) and Pyrotell-T (lot 42-13-575) from Associates of Cape Cod (ACC), Woods Hole, Mass.; LAL (lot 2L0860) from Whittaker Bioproducts, Walkersville, Md.; and LAL (lot 29157-51) from Kabi Vitrum Diagnostica, Stockholm, Sweden.

Raw amebocyte lysates from *Limulus polyphemus* (obtained from the Marine Biological Laboratory, Woods Hole, Mass.) and *Tachypleus tridentatus* (collected from the Beibu

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Gulf of the South China Sea) were prepared as follows. Hemolymph was collected by cardiac puncture, placed in pyrogen-free plastic tubes, and centrifuged at 5,000 × *g* for 30 min at 4°C. The cell-free hemolymph was decanted, and LAL reagent water equivalent to approximately 6 volumes of amebocyte precipitate was added. The amebocytes were disrupted by vigorous shaking by hand for 5 min. After being kept at 4°C overnight, the lysate was centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatant was stored at −30°C. Chloroform-treated TAL was prepared essentially as described previously (22).

Endotoxin standard. The control standard endotoxin (CSE) was NP-3, which is a purified preparation of lipopolysaccharide from *Salmonella abortus equi* (Pyroquant Diagnostik, Walldorf, Germany), and its potency was confirmed to be 10 EU/ng by comparison with USP reference standard endotoxin EC-5.

Glucan preparations. Laminarin isolated from *Laminaria digitata* was obtained from Sigma (St. Louis, Mo.). Highly purified curdlan, a water-insoluble, carboxymethylated powder, 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.

MAb against peptide C. *Limulus* peptide C, consisting of 28 amino acid residues (7, 23), was synthesized by the solid-phase "Fmoc" method (18) and conjugated in a molar ratio of 2:1 to purified protein derivative (State Serum Institute, Copenhagen, Denmark) with 0.1% glutaraldehyde (vol/vol) as the coupling reagent. The conjugate was adsorbed to an aluminum hydroxide suspension (2 mg/ml in normal saline) and injected intraperitoneally (0.5 ml per mouse, equivalent to 25 μg of conjugated peptide) into female CF1 × BALB/c mice. Booster doses were given at 2-week intervals. When high antibody titers were found by an ELISA with biotinylated peptide linked to avidin-coated microtiter plate, spleen cells were fused by a standard procedure (4) with myeloma cells from line X63/Ag8.6.5.3. Culture supernatants were harvested from hybridomas isolated after repeated clonings by limiting dilution and screened by the ELISA as described above. Selected MAbs against peptide C were purified from culture supernatant by protein A–Sepharose CL-4B affinity chromatography (Pharmacia, Uppsala, Sweden).

Conjugation of MAb to horseradish peroxidase through a biotin-avidin bridge (MAb-baHRP conjugate). One milliliter of purified MAb (1 mg/ml) was dialyzed against 0.1 M $NaHCO_3$ overnight at 4°C and mixed with 5 μl of *N*-hydroxysuccinimidobiotin (40 mg/ml in *N*,*N*-dimethylformamide) (Sigma). After being rotated at room temperature for 2 h, the mixture was dialyzed against phosphate-buffered saline (PBS) (pH 7.3) overnight at 4°C. The biotinylated MAb was then mixed with an equal volume of streptavidin-horseradish peroxidase (HRP) conjugate (Zymed Laboratories, San Francisco, Calif.) and two volumes of glycerol and was stored at 4°C.

Immunoblotting of amebocyte lysates. Endotoxin-reacted amebocyte lysates were prepared by incubating raw lysates with an equal volume of CSE (10 ng/ml) for 1 h at 37°C. Raw and endotoxin-reacted amebocyte lysate samples were then diluted 10-fold in the sample buffer (0.06 M Tris-HCl [pH 6.8] containing 10% glycerol and 2% sodium dodecyl sulfate [SDS]) with or without 5% 2-mercaptoethanol and boiled for 3 min before being subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This was carried out with 12% gels, essentially as described by Laemmli (10), with biotinylated molecular weight markers (Bio-Rad Laboratories, Richmond, Calif.). The separated proteins were electroblotted onto nitrocellulose paper (Schleicher & Schuell, Dassel, Germany), which was then blocked with PBS containing 0.5% Tween 20 and 0.5 M NaCl. The nitrocellulose paper was incubated for 1 h with MAb-baHRP conjugate diluted 1:2,000 in PBS containing 0.05% Tween 20 and streptavidin-HRP (Zymed) diluted 1:4,000 in the same buffer. After the paper was washed with the same buffer, the substrate solution (3,3-diaminobenzidine tetrahydrochloride in buffer, pH 7.0) (KemEnTec, Copenhagen, Denmark) was added, and color development was terminated by transferring the nitrocellulose paper to distilled water.

***Limulus* peptide C ELISA.** Twenty-microliter samples of LAL diluted fourfold in LAL buffer (0.1 M Tris-HCl [pH 8.0] containing 0.15 M NaCl and 0.02 M $MgCl_2$) were added to the wells of a pyrogen-free microplate and mixed with equal volumes of test samples or standards. The plate was then placed in a hot-plate incubator at 37°C for 20 to 40 min, depending on the desired sensitivity. The following two types of ELISA were performed at room temperature after termination of the reaction.

(i) Noncompetitive ELISA. The reaction was stopped by adding 200 μl of 50 mM NaOH to each well, and 20-μl aliquots of the mixtures were transferred to a microtiter plate (Maxisorp; Nunc) to which 50 mM NaOH (80 μl per well) had previously been added. After incubation for 30 min, the plate was washed four times with washing buffer (PBS containing 0.5 M NaCl and 0.05% Triton X-100, pH 7.3), and 100 μl of MAb-baHRP conjugate at 1:2,000 in dilution buffer (washing buffer containing 1% bovine albumin) was added to each well. After incubation for 30 min, the plate was washed four times, and 100 μl of the substrate solution (*o*-phenylenediamine dihydrochloride [0.4 mg/ml] and 0.014% H_2O_2 in 0.1 M sodium phosphate-citric acid buffer, pH 5.0) was added. The color development was stopped by the addition of 150 μl of 1 M H_2SO_4, and the plate was read at 490 nm with a microplate reader (Molecular Devices Inc., Menlo Park, Calif.).

(ii) Competitive ELISA. Microtiter plates were coated (100 μl per well) with rabbit anti-mouse immunoglobulins (Z-109; Dakopatts, Glostrup, Denmark) diluted 1:2,000 in 10 mM Tris-HCl buffer (pH 8.6). The LAL-endotoxin reaction was stopped by the addition of 100 μl of MAb-baHRP conjugate diluted 1:1,000 in washing buffer containing 10 mM benzamidine (Sigma) to each well. The plate was shaken vigorously for about 5 min on a shaking platform, and 100 μl of the contents of each well was then transferred to the precoated microtiter plate, which was washed four times immediately before use. After incubation for 30 min, the plate was washed four times, and the substrate solution (100 μl per well) was added. Color development was stopped and the plate was read as described above.

Preparation and pretreatment of plasma. Blood from human and laboratory animals 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 precipitation of plasma to remove factors that interfere with LAL was carried out essentially as described by Obayashi (15) with one minor modification: the neutralized supernatant was further diluted 1:2 in the LAL reagent water.

Other LAL assays. The gel-clot and chromogenic assays were performed according to the manufacturers' instructions. The chromogenic substrate from the Whittaker chro-

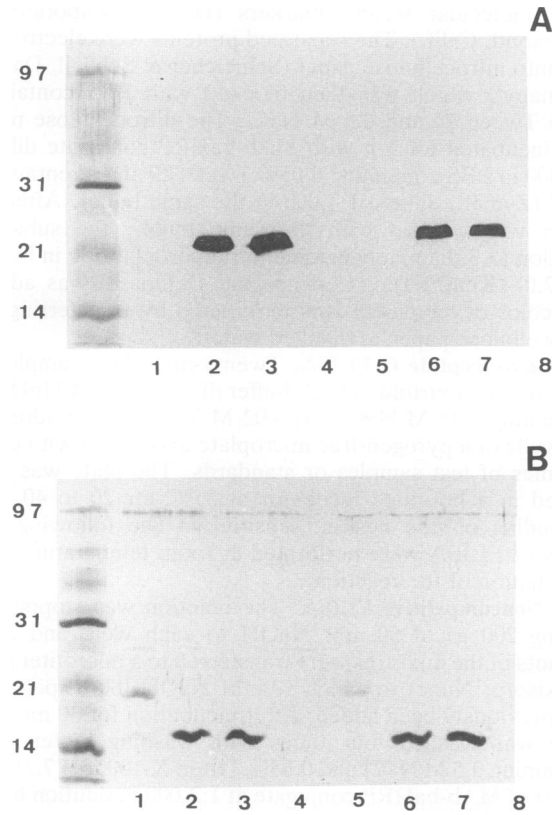

FIG. 1. Immunoblotting of LAL (lanes 1 to 4) and TAL (lanes 5 to 8) before (lanes 1 and 5) and after (lanes 2 to 4 and 6 to 8) reaction with endotoxin, detected with the biotinylated MAb against synthetic *Limulus* peptide C. Lanes 2 and 6 contain samples of suspensions of endotoxin-reacted lysates. Lanes 3 and 7 contain samples of precipitates of the reacted lysates. Lane 4 and 8 contain samples of supernatants of the reacted lysates. The left lane shows biotinylated molecular weight markers; numbers are molecular weights in thousands. (A) Unreduced samples; (B) reduced samples.

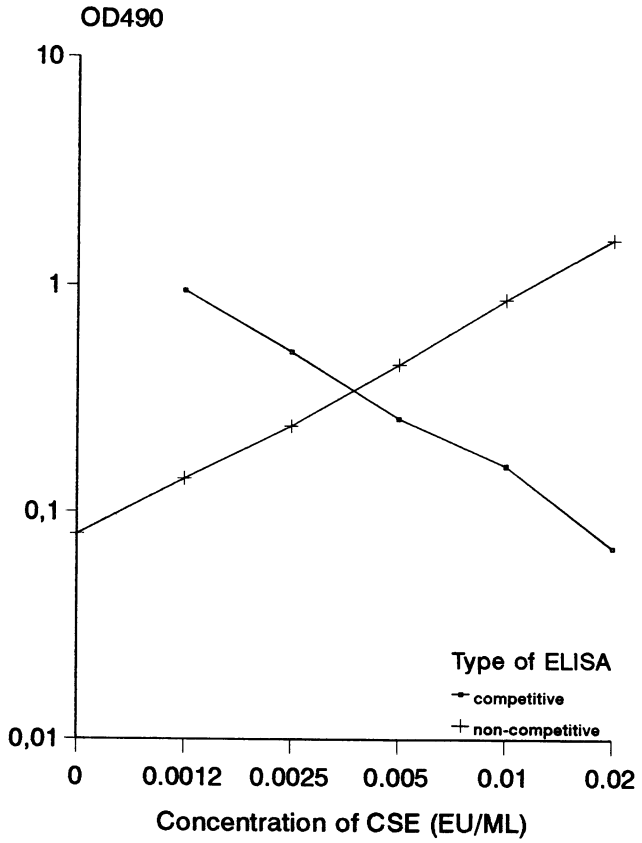

FIG. 2. Standard curves of the noncompetitive and competitive *Limulus* peptide C ELISAs, plotted on a double logarithmic scale. LAL (ACC; lot 42-99-541) was reacted with CSE for 40 min at 37°C.

mogenic LAL assay kit was used for all the chromogenic assays in this study.

RESULTS

Immunoblotting of *Limulus* amebocyte lysates. Figure 1 shows SDS-PAGE immunoblots of amebocyte lysates before and after reaction with endotoxin, as detected with the MAb-baHRP conjugate. Without 2-mercaptoethanol reduction, reacted lysates and precipitates of centrifuged reacted lysates gave prominent reactive bands at the 21-kDa position, while unreacted lysates and supernatants of centrifuged reacted lysates gave no reactive band. With reduction, those reactive bands occurred at the 16-kDa position. The weak band at the 21-kDa position in the reduced unreacted lysates represents coagulogen, as was confirmed by its reaction with MAb against coagulogen, while the prominent band in the endotoxin-reacted samples represents a product with strong peptide C-like immunoreactivity. No band corresponding to free peptide C was observed. Figure 1 also shows the cross-reaction of MAb-baHRP with the peptide C-like immunoreactivity of reacted TAL.

Standard curves of the *Limulus* peptide C ELISA. Figure 2 shows the standard curves for the two types of ELISA. In the noncompetitive ELISA, in which the generation of peptide C-like immunoreactivity was measured directly with the MAb-baHRP conjugate, the optical density (OD) was directly proportional to the concentration of endotoxin. In the competitive ELISA, in which the generation of peptide C-like immunoreactivity was measured indirectly by its inhibition of binding of MAb-baHRP conjugate to the wells, the OD decreased with increasing endotoxin concentrations. Similar reaction plots with different sensitivities were obtained with commercial LALs and TALs, and reaction plots for endotoxins from different bacterial sources were parallel (data not shown). In the noncompetitive assay, total protein concentrations of >0.1 mg/ml in the samples interfered with the nonspecific binding of peptide C-like immunoreactivity to the microtiter wells, leading to underestimation of endotoxin concentrations, whereas no such interference was observed in the competitive assay. The turbidity or color of the original sample does not interfere with either assay, as it is washed out during the procedure.

The intra-assay coefficient of variation, estimated by determining the endotoxin content of a solution giving an OD value close to the midpoint of the standard curve, was 10.3% (n = 6) for the noncompetitive assay and 13.8% (n = 6) for the competitive assay.

Effects of reaction time and dilution of the LAL and TAL. The sensitivity of the assay with respect to endotoxin increased with increasing time of the LAL-endotoxin reaction (Fig. 3), and a 1:4 dilution of LAL or TAL was found to give the best sensitivity at the reaction times shown (Fig. 4

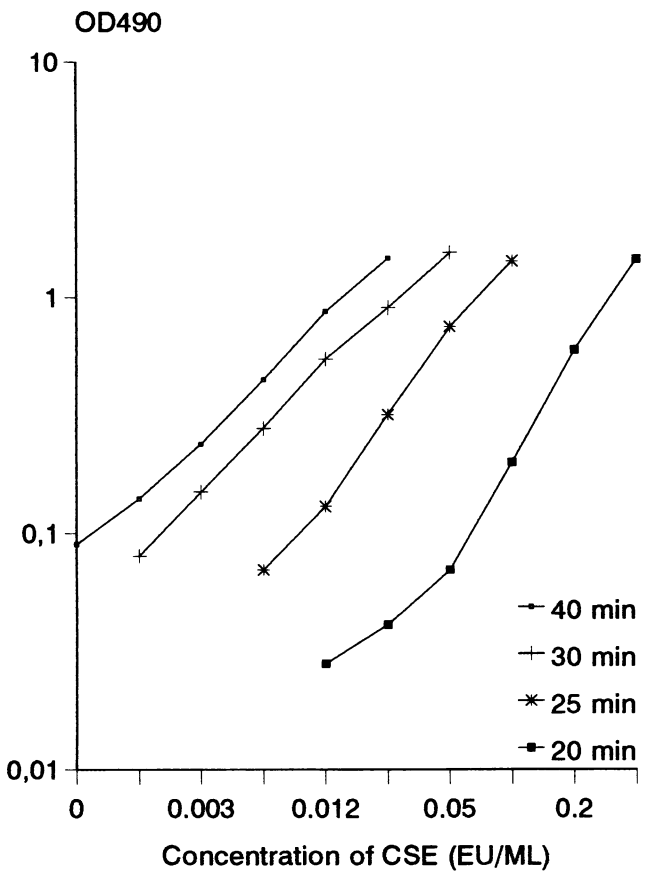

FIG. 3. Effect of time of reaction of CSE with LAL on the sensitivity of the noncompetitive peptide C ELISA, plotted on a double logarithmic scale. The lysate (ACC; lot 42-99-541) was diluted in LAL buffer (see Materials and Methods) by a factor of 5.

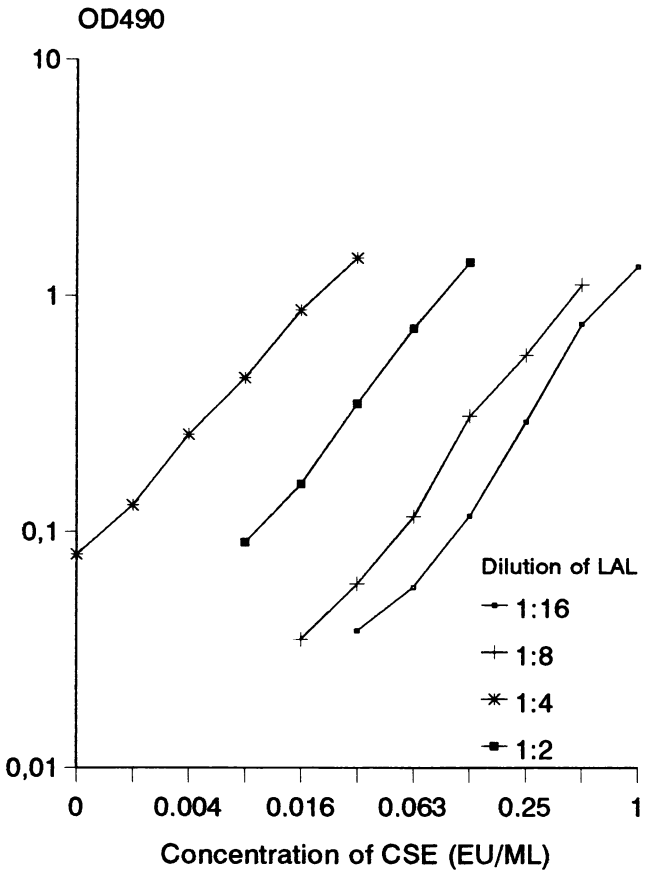

FIG. 4. Effect of dilution of LAL on the sensitivity of the noncompetitive peptide C ELISA, plotted on a double logarithmic scale. The lysate (ACC; lot 42-99-541) was reacted with CSE for 40 min at 37°C.

and 5). With further dilution, an increased susceptibility to specimen-related interference was noted. The linear portion of the assay curve obtained with TAL covered a wider range of endotoxin concentrations than that obtained with LAL. The sensitivity obtained at 37°C could be achieved at room temperature (22°C) by extending the LAL-endotoxin reaction time by about 30%.

Comparison of sensitivities of different *Limulus* assays. Table 1 compares the sensitivities of the *Limulus* peptide C ELISA, the gel-clot assay, and the chromogenic assay, using different commercial LALs and TALs. The sensitivity of the gel-clot assay is given by the minimum concentration of CSE that produces a gel-clot, and that of the other assays is given by the minimum concentration of CSE to generate an OD value significantly higher than that of the LAL reagent water control. The *Limulus* peptide C ELISA was more sensitive than the chromogenic or the gel-clot assay. When raw LAL or TAL was used, the ELISA could detect less than 1/50 of the minimum concentrations detected by the other assays. Chloroform extraction of the LAL increased the sensitivities of the gel-clot and chromogenic assays but was unnecessary for the *Limulus* peptide C ELISA.

Stability of LAL and TAL in solution. Table 2 shows the effects of chloroform extraction or the addition of dimethyl sulfoxide (DMSO) on the stability of LAL in solution at 4°C. Chloroform extraction of LAL has been used to increase the sensitivities of the gel-clot and chromogenic assays but was shown here to bring about a dramatic reduction of the stability of LAL in solution. Addition of DMSO to the LAL solution to a concentration of 10% (vol/vol) increased the stability of raw LAL but did not appreciably alter the stability of chloroform-treated LAL. Similar effects of chloroform and DMSO on the stability of TAL in solution were also observed.

Endotoxins in normal plasma. By using the PCA method to pretreat plasma samples, the endotoxin levels in normal human, mouse, rabbit, and guinea pig plasmas were found to be either below or close to the detection limit (0.01 EU/ml of plasma), while those of patients with gram-negative sepsis were generally above 0.1 EU/ml of plasma (data not shown).

Sensitivities of different LAL and TAL preparations to β-glucans. Using the *Limulus* peptide C ELISA, we tested the sensitivities of several commercial and raw LAL and TAL preparations to curdlan, a carboxymethylated β-glucan, and laminarin, a natural β-glucan. Different LAL and TAL preparations were classified as chloroform-extracted and nonextracted forms. Table 3 shows that (i) all the LAL and TAL preparations reacted with curdlan, (ii) the chloroform-treated preparations of LAL and TAL reacted with both curdlan and laminarin, and (iii) the raw LAL and TAL did not react with laminarin.

When raw TAL or LAL was added to commercial chloroform-treated LAL (from ACC) in equal amounts, no change in the reactivity of the commercial LAL to laminarin

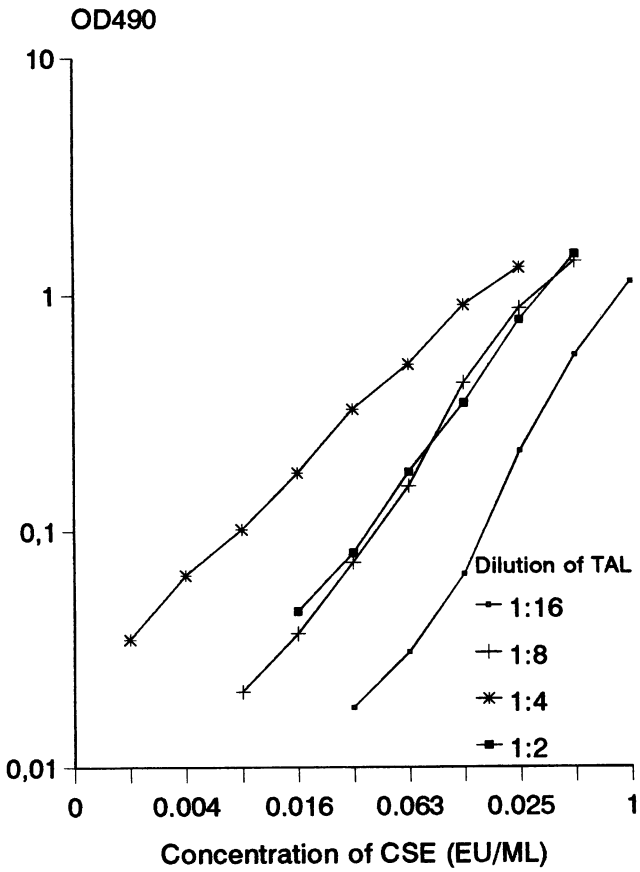

FIG. 5. Effect of dilution of TAL on the sensitivity of the noncompetitive peptide C ELISA, plotted on a double logarithmic scale. The raw TAL (lot 101092) was reacted with CSE for 30 min at 37°C.

was observed, suggesting that there was no laminarin inhibitor in the raw preparations. Interestingly, the raw TAL or LAL became reactive to 50 pg of laminarin per ml when as little as 10% of the commercial LAL was added, indicating that there was a component in the latter that was responsible for the reactivity. Other chloroform-treated LALs had a similar effect on the reactivity of raw TAL or LAL to laminarin.

TABLE 1. Comparison of the sensitivities of different endotoxin assays by using LAL or TAL

LAL or TAL source	Detection limit[a] (EU/ml) with:		
	Gel-clot assay	Chromogenic assay	ELISA
ACC LAL lot 42-99-541[b]	0.06	0.025	0.002
ACC LAL lot 42-133-575[b]	0.06	0.031	0.001
Kabi Vitrum LAL lot 29157-51[b]	0.06	0.012	0.006
Whittaker Bioproducts LAL lot 2L0860[c]	Not clottable	0.012	0.004
Raw LAL lot 051291[c]	0.50	0.250	0.002
Raw TAL lot 101092[c]	0.50	0.250	0.004
Raw TAL lot 150293[c]	0.50	0.125	0.002

[a] For CSE under conditions optimized for each assay.
[b] Chloroform-treated preparation.
[c] Preparation without chloroform treatment.

TABLE 2. Effects of chloroform and DMSO treatments on the stability of LAL in solution

Days of storage at 4°C	Detection limit[a] (EU/ml)			
	Chloroform-treated LAL[b]	Chloroform-treated LAL[b] + 10% DMSO	Raw LAL	Raw LAL + 10% DMSO
1	0.004	0.004	0.004	0.004
2	0.004	0.004	0.004	0.004
3	0.008	0.004	0.004	0.004
4	0.032	0.008	0.004	0.004
5	>0.1	0.016	0.004	0.004
10		>0.1	0.004	0.004
30			0.008	0.004
60			0.016	0.004
90			0.032	0.008

[a] For CSE incubated with LAL diluted 1:4 for 30 min (40 min for LAL containing DMSO).
[b] From ACC, lot 42-99-541.

DISCUSSION

The molecular mechanism of gel formation in LAL and TAL has been extensively studied by Iwanaga et al. (7, 23). Coagulogen consists of a single basic polypeptide chain with a calculated molecular mass of 19.7 kDa. It contains three regions, the A chain, peptide C, and the B chain, of 18, 28, and 129 amino acid residues, respectively. On gelation, peptide C is released and the gel consists of two chains of A and B joined by two disulfide bridges. Our immunoblotting analyses show that the peptide-C immunoreactivity of coagulogen is formed by the reaction of endotoxin with the LAL. Since there is no apparent loss of molecular weight of coagulogen and no free peptide C-reactive band, it seems that peptide C is not split off from coagulogen under our experimental conditions. The observed band with peptide C immunoreactivity may represent an intermediate consisting of peptide C linked to the B chain, as suggested by Tagaki et al. (23). The intermediate (or peptide C-containing fragment)

TABLE 3. Sensitivities of different LALs or TALs to curdlan and laminarin in the noncompetitive peptide C ELISA

LAL or TAL source	Detection limit[a] (ng/ml)	
	Curdlan	Laminarin
ACC LAL lot 42-99-541[b]	0.05	0.03
Kabi Vitrum LAL lot 29157-51[b]	0.50	0.50
TAL lot 220992[b]	0.03	0.01
Whittaker Bioproducts LAL lot 2L0860[c]	1.00	Inactive
Raw LAL lot 051291[c]	0.25	Inactive
Raw TAL lot 150293[c]	0.05	Inactive

[a] Under conditions optimized for each lysate.
[b] Chloroform-treated preparation.
[c] Preparation without chloroform treatment.

probably occurs in a polymerized form, since it is absent in the supernatant and is found only in the insoluble gel of endotoxin-reacted lysates.

No reaction between MAb-baHRP and unreduced coagulogen was detected. Reduction of coagulogen with 2-mercaptoethanol seems to lead to some exposure of the peptide C epitope, allowing a weak reaction to occur. The use of diluted NaOH in the noncompetitive ELISA serves two functions, that of terminating the LAL-endotoxin reaction and that of increasing the binding of the peptide C-containing fragment to the microtiter plate (data not shown). In the competitive ELISA, benzamidine, a serine protease inhibitor (14), is used to stop the LAL-endotoxin reaction.

The sensitivity of the *Limulus* peptide C ELISA was found to depend on the dilution of the LAL reagent and the incubation time of the LAL-endotoxin reaction. The detection limit of the *Limulus* peptide C ELISA for endotoxin is mainly restricted by the endotoxin content of LAL. When no limit was set for the LAL-endotoxin reaction time, eightfold-diluted raw LAL gave greater sensitivity than fourfold-diluted LAL, which in turn gave greater sensitivity than twofold-diluted LAL. One explanation of this result could be that when LAL was diluted, the endotoxin concentration of the LAL, and hence the assay background, was reduced accordingly. Another explanation could be that LAL contains endogenous inhibitors of the LAL-endotoxin reaction, whose effects are reduced by dilution.

The stability of all LAL assays depends on the stability of the LAL reagents. Most commercial LAL reagents have been extracted with chloroform, a method known to improve the sensitivities of the gel-clot and chromogenic assays by at least a factor of 5 (22). However, chloroform-treated LAL was shown to be much less stable than raw LAL in solutions. Inclusion of 10% DMSO was found to increase the stability of raw LAL and TAL in solution. Our results suggested that DMSO produced a reversible inhibition of LAL reactivity and a slow inactivation of endotoxin contaminating the LAL that together might promote the stability of LAL solutions. No significant differences between endotoxin concentrations measured with raw LAL or TAL and those measured with DMSO-containing raw LAL or TAL were observed, showing that the final DMSO concentration in the reaction mixture was without effect on the endotoxin in the samples.

The specificity of the LAL test has been questioned by several studies that have demonstrated reactivity of LAL to certain β-glucans. Laminarin has been shown to be one of the most potent LAL-reactive β-glucans (1). Our results with the *Limulus* peptide C ELISA confirm the finding of Söderhall et al. (21), who reported reactivity with curdlan but not with laminarin when an LAL reagent that had not been extracted with chloroform was used and the contamination of laminarin by endotoxin was eliminated. Roslansky and Novitsky (20) reported some reactivity of raw LAL and non-chloroform-treated LAL with laminarin at concentrations of 10 to 100 μg/ml. Our findings were similar, provided that the laminarin was not pretreated with NaOH, a procedure to inactivate endotoxin. Furthermore, we observed that laminarin totally blocked the β-glucan activation pathway at concentrations of >1 μg/ml. These results suggest that the reactivity observed by Roslansky and Novitsky (20) was probably due to endotoxin contamination of the laminarin preparation.

The mechanism by which chloroform treatment renders the LAL or TAL reactive to laminarin is not known. Chloroform extraction has been found to increase LAL sensitivity to both endotoxin and β-glucan, and the removal of lipoproteins that might function as inhibitors has been tentatively proposed as the underlying mechanism (20, 22). Since the addition of raw LAL or TAL to commercial chloroform-treated LAL did not affect the sensitivity of the latter to laminarin (unpublished observation), it seems unlikely that there was any laminarin inhibitor in the raw LAL or TAL that might explain their lack of reactivity.

TAL has been shown to be biochemically similar and functionally identical to LAL (7). In this study, we have demonstrated that the peptide C-containing fragments from LAL and TAL are identical in molecular size and comparable in immunoreactivity. The two lysates show similar sensitivities to endotoxin, similar reactivities to curdlan and laminarin after chloroform extraction, and no reactivity to laminarin in their raw state. Thus, TAL can be used as a substitute for LAL to detect endotoxin both in assays previously described and in the ELISAs described here. The latter present a significant advantage, not only because of their higher sensitivity but also because they reduce LAL or TAL consumption to 5% of that of the other assays. This is an important consideration in view of the increasing use of these reagents and the diminishing populations of rare horseshoe crab species from which they are obtained.

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