Discrimination between Endotoxin and (1→3)-β-D-Glucan Using Turbidimetric Kinetic Assay with *Limulus* Amebocyte Lysate

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A procedure for the *Limulus* amebocyte lysate (LAL) test discriminating between endotoxin and (1→3)-β-D-glucan based on the turbidimetric kinetic method was proposed. Endotoxin and (1→3)-β-D-glucan, which are elicitors of the activation of LAL, showed different reaction courses with this lysate. To analyze the difference in the reactions, two parameters, the maximum differential coefficient of the reaction (D_{max}) and the reaction time required to obtain D_{max} (T_p) were defined. The logarithmic plottings of T_p *versus* D_{max} (T_p–D_{max} plot) discriminated between endotoxin and (1→3)-β-D-glucan. Endotoxin was measured with a standard curve plotting logarithmic endotoxin concentration *versus* D_{max} (ET–D_{max} plot). The endotoxin calculated from D_{max} was less influenced by (1→3)-β-D-glucan than that calculated from the usual gelation time. A small amount of endotoxin in a sample could be concealed by the addition of polymyxin B, which inhibited the activation of LAL by endotoxin. (1→3)-β-D-glucan was measured without being affected by the presence of a small amount of endotoxin using LAL with polymyxin B. The following procedure is proposed as a LAL test to discriminate between endotoxin and (1→3)-β-D-glucan.

(1) Identify the main substance (endotoxin or (1→3)-β-D-glucan) triggering the activation of LAL using the T_p–D_{max} plot. (2) Use the appropriate method to measure the main substance: the ET–D_{max} plot for endotoxin or the LAL with polymyxin B for (1→3)-β-D-glucan.

Keywords endotoxin; (1→3)-β-D-glucan; *Limulus* amebocyte lysate; turbidimetric kinetic method; reaction course; differential coefficient; T_p–D_{max} plot; ET–D_{max} plot; polymyxin B

Introduction

Limulus amebocyte lysate (LAL) is now often used for the detection of bacterial endotoxins in pharmacology, particularly for in-process and final-release bacterial endotoxins tests of medical devices and parenteral drugs. The LAL test is based on the coagulation of a hemocyte lysate from horseshoe crabs by bacterial endotoxins.[1] The coagulation is a sequence of enzymatic reactions and involves two key proteins, proclotting enzyme and coagulogen. The proclotting enzyme is activated through the sequential activation of protease zymogens triggered by endotoxins.[2] Then the clotting enzyme (the activated proclotting enzyme) splits the coagulogen into coagulin, which interacts with itself to form a gel.[3] The LAL test has been improved in sensitivity and methodology by development of several superior techniques, such as the chromogenic method[4] and the turbidimetric kinetic method.[5]

The usual purpose of the LAL test is the detection of endotoxin; since it is one of the most pyrogenic substances. The LAL test has long been thought to detect only gram-negative bacterial endotoxins. However, biochemical studies have shown that (1→3)-β-D-glucan also activates the coagulation system in LAL by a mechanism different from that by endotoxin.[2] Because the LAL test for the detection of endotoxin can be disturbed by (1→3)-β-D-glucan, a method to discriminate between the two in the LAL test has been needed. Obayashi *et al.*[6] have developed an endotoxin-specific assay based on recombination of the *Limulus* coagulation factors. This method requires fractionation and recombination of the coagulation factors in LAL. These procedures require strict pyrogen-free processing.

(1→3)-β-D-glucan commonly exists in fungi and plants, but its biological effects are not sufficiently clear. The ability to detect (1→3)-β-D-glucan may be useful so that the biological activity can be investigated.

We have studied the activation kinetics of LAL extensively using the turbidimetric kinetic method, and found that the reaction course of LAL with (1→3)-β-D-glucan is different from that of LAL with endotoxin[7]; this led us to further analyze these reactions. This study introduces a method for the analysis of the reaction kinetics of LAL with endotoxin and (1→3)-β-D-glucan. We propose a procedure for the LAL test using turbidimetric kinetics that discriminates between these two substances.

Materials and Methods

Reagents LAL reagent (manufactured by Associates of Cape Cod, Inc., lot No. 99-65-408), (1→3)-β-D-glucan (curdlan), and polymyxin B sulfate were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Carboxymethylated (1→3)-β-D-glucan (CM-curdlan) was the gift of Dr. A. Kakinuma, Takeda Chemical Industries, Ltd., Osaka, Japan. U.S. Standard Endotoxin lot No. EC-5 was used as endotoxin. Water for injection (WFI) was purchased from Otsuka Pharmaceuticals, Ltd., Tokyo, Japan.

Equipment A Toxinometer ET-201 (Wako) was used for the turbidimetric kinetic assay. This instrument incorporates one control module and four analysis modules. Up to 64 samples were incubated at 37 °C and the turbidity change of each sample was measured simultaneously at the wavelength of 660 nm. The data from the Toxinometer was collected and analyzed on a personal computer (NEC PC-9801 VX) with Labosoft-ET (Wako), a data acquisition program for this machine. Glassware and spatulas were rendered endotoxin-free by being baked at 250 °C for 2 h.

Principle of the Assay Figure 1 shows the definitions of T_g, D_{max}, and T_p. The gelation time T_g and the transmittance ratio $R(t)$ were previously defined for the turbidimetric kinetic assay by the Toxinometer.[7] The differential coefficient of $R(t)$ was obtained by the method of Savitzky and Golay,[8] and designated as $D(t)$. D_{max} was defined as the maximum value of $D(t)$. T_p was defined as the reaction time required to obtain D_{max}.

Assay of Endotoxin and (1→3)-β-D-Glucan Curdlan, (1→3)-β-D-glucan obtained from the culture filtrate of *Alcaligenes faecalis* var. *myxogenes* IFO 13140, was dissolved in 50 mM sodium hydroxide, and then diluted with WFI. U.S. Standard Endotoxin was reconstituted with 5 ml WFI, and 2000 EU/ml (EU: endotoxin units) endotoxin solution was obtained. Endotoxin and CM-curdlan were dissolved and diluted with WFI.

A 0.1-ml sample of test fluid was added to 0.1 ml of LAL reconstituted with WFI in a 10×75 mm glass tube. The test tube was set on the

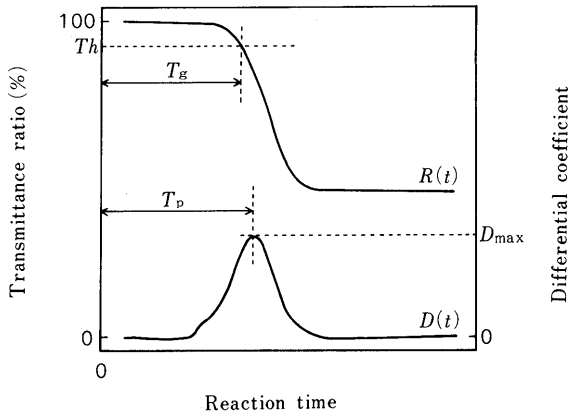

Fig. 1. Definition of T_g, D_{max}, and T_p

$R(t)$, transmittance ratio of the reaction mixture; Th, threshold value of $R(t)$; T_g, gelation time; $D(t)$, differential coefficient of $R(t)$; D_{max}, maximum value of $D(t)$; T_p, reaction time required to obtain D_{max}.

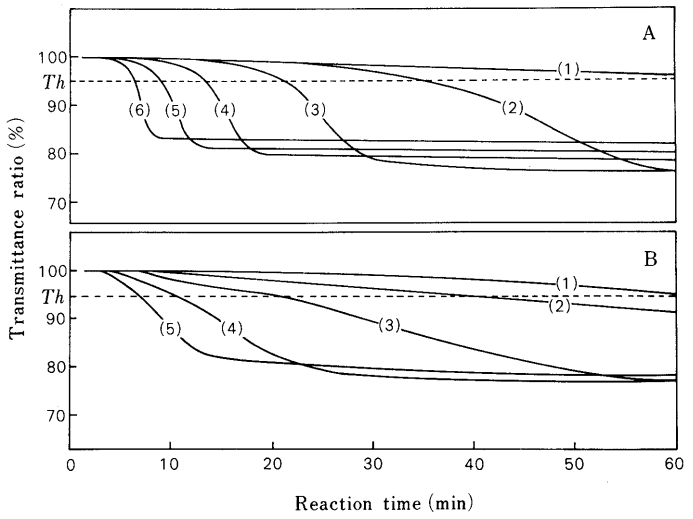

Fig. 2. Courses of Reactions of Endotoxin (A) and Curdlan (B) with LAL

Endotoxin concentration of each sample in Fig. 2A was as follows: (1), none; (2), 0.05 EU/ml; (3), 0.5 EU/ml; (4), 5 EU/ml; (5), 50 EU/ml; (6), 500 EU/ml. Curdlan was used as (1→3)-β-D-glucan. Curdlan concentration of each sample in Fig. 2B was as follows: (1), none; (2), 0.2 ng/ml; (3), 2 ng/ml; (4), 20 ng/ml; (5), 200 ng/ml.

Toxinometer after being vortexed for a few seconds, and the measurement was started. The gelation time used in this study was defined as the reaction time required to obtain a 5% decrease in the transmittance of the sample.

Results

T_p–D_{max} Plot Figure 2 shows the courses of reactions of endotoxin and curdlan with LAL. The reaction courses differed with the reaction between endotoxin and LAL having a faster rate of turbidity change, and that between curdlan and LAL having an earlier reduction of the transmittance ratio.

To clarify the difference, we attempted to plot T_p *versus* D_{max} logarithmically. Endotoxin and CM-curdlan were plotted on different lines (Fig. 3), with mixtures of endotoxin and curdlan falling between the two lines (Fig. 4). Higher concentrations of both endotoxin and (1→3)-β-D-glucan tended to be plotted at higher D_{max}, but D_{max} reflected the concentration of endotoxin more than that of (1→3)-β-D-glucan. Curdlan (Fig. 4) and CM-curdlan (Fig. 3) were plotted on lines that looked similar.

ET–D_{max} Plot The concentration of endotoxin was correlated with D_{max} (Fig. 5), therefore, endotoxin was

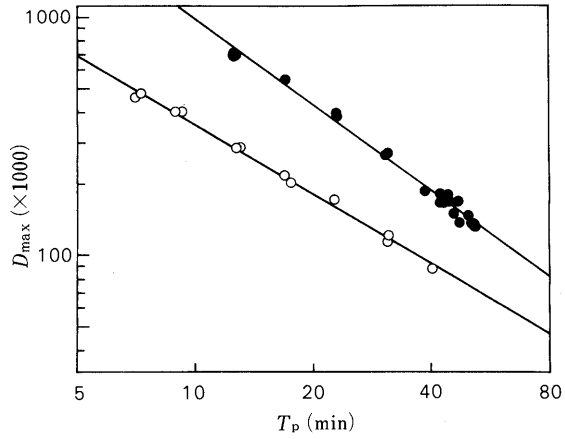

Fig. 3. T_p–D_{max} Plot of Endotoxin and CM-Curdlan

D_{max} and T_p were obtained from the reaction course of each sample. Endotoxin (●) was assayed in the range of 0.03 to 10 EU/ml. CM-curdlan (○) was assayed in the range of 0.5 to 100 ng/ml. The regression line of endotoxin was $\log(D_{max}) = -1.20(\log(T_p)) + 4.2$ ($r = 0.995$). The regression line of CM-curdlan was $\log(D_{max}) = -0.975(\log(T_p)) + 3.52$ ($r = 0.996$). Higher concentrations of endotoxin and CM-curdlan were plotted at a point of higher D_{max} and lower T_p on each regression line.

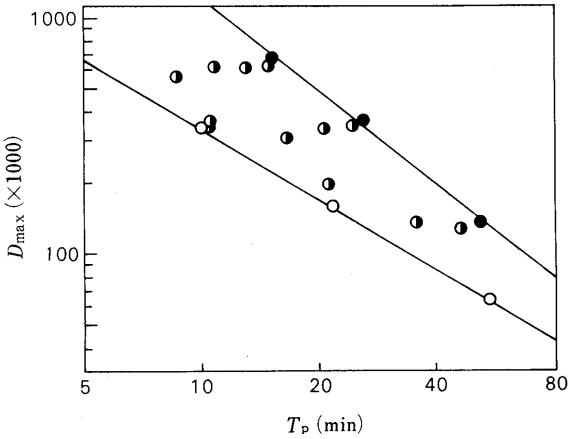

Fig. 4. T_p–D_{max} Plot of Mixture of Endotoxin and Curdlan

D_{max} and T_p were obtained from the reaction course of each sample. The concentrations of endotoxin in the samples were 0.025, 0.25, and 2.5 EU/ml. The concentrations of curdlan were 0.1, 1, and 10 ng/ml. The solid lines in the figure are the regression lines of endotoxin (●) and curdlan (○). The mixture (◑) of endotoxin and curdlan was constructed by a combination of the endotoxin sample and the curdlan sample.

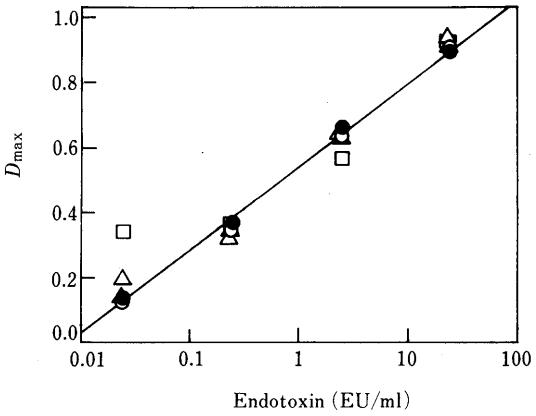

Fig. 5. Effects of Curdlan on Endotoxin Standard Curve

Endotoxin dilutions containing several concentrations of curdlan were assayed with the Toxinometer ET-201. Curdlan concentrations were 0.1 ng/ml (○), 1 ng/ml (▲), 10 ng/ml (△), and 100 ng/ml (□). Endotoxin concentrations were 0.02, 0.2, 2, 20 EU/ml. The solid line in the figure is the regression line of endotoxin only (●).

TABLE I. Effects of CM-Curdlan on Endotoxin Measurement Based on ET–D_{max} Plot[a] and ET–T_g Plot[b]

Sample		ET–D_{max} plot		ET–T_g plot	
Endotoxin (EU/ml)	CM-curdlan (ng/ml)	D_{max} (×1000)	Endotoxin (EU/ml)	T_g (min)	Endotoxin (EU/ml)
0.125	None	253	0.127	22.9	0.132
0.125	0.05	256	0.131	21.6	0.170
0.125	0.5	241	0.112	17.2	0.480
0.125	5	263	0.141	11.4	3.91
0.125	50	422	0.707	6.4	149

a) Endotoxin concentration was calculated from D_{max} with a plot of logarithmic endotoxin concentration *versus* D_{max} (ET–D_{max} plot) as an endotoxin standard curve. *b*) Endotoxin concentration was calculated from T_g with a logarithmic plot of endotoxin concentration *versus* T_g (ET–T_g plot) as an endotoxin standard curve. This plot is commonly used for the measurement of endotoxin with the Toxinometer because of its simplicity and precision. The endotoxin solution (0.125 EU/ml) was spiked with several concentrations of CM-curdlan and assayed with the Toxinometer ET-201.

measured using a plot of endotoxin concentration *versus* D_{max}. When curdlan was present at 10 or 100 ng/ml, the calibration curve of endotoxin was disturbed at lower concentrations (Fig. 5).

Table I summarizes the effects of CM-curdlan on the measurement of endotoxin using D_{max} and T_g. The endotoxin concentration calculated from T_g was affected by CM-curdlan at concentrations of 0.05 to 50 ng/ml. The endotoxin concentration calculated from D_{max} was almost unaffected by 5 ng/ml CM-curdlan or less.

Polymyxin B Studies Figure 6 shows the effects of polymyxin B on the reactions of endotoxin and CM-curdlan with LAL. Polymyxin B partially inhibited the reaction between 0.2 EU/ml endotoxin and LAL (Fig. 6A), but did not affect the reaction between CM-curdlan and LAL (Fig. 6B).

Figure 7 shows the effects of polymyxin B on the measurement of CM-curdlan using T_g. The dose–response curve of CM-curdlan was lowered by the addition of 0.125 EU/ml endotoxin (dashed line in Fig. 7). However, the addition of 0.2 mg/ml polymyxin B neutralized the effect of 0.125 EU/ml of spiked endotoxin on the assay of CM-curdlan.

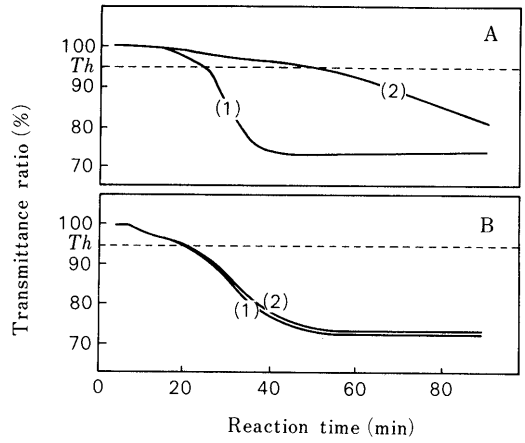

Fig. 6. Effects of Polymyxin B on the Reaction Courses of Endotoxin (A) and CM-Curdlan (B) with LAL

The sample assayed in Figs. 6A and 6B contained 0.2 EU/ml endotoxin and 1 ng/ml CM-curdlan, respectively. The concentration of polymyxin B in each sample in both was: (1), none; (2), 0.2 mg/ml.

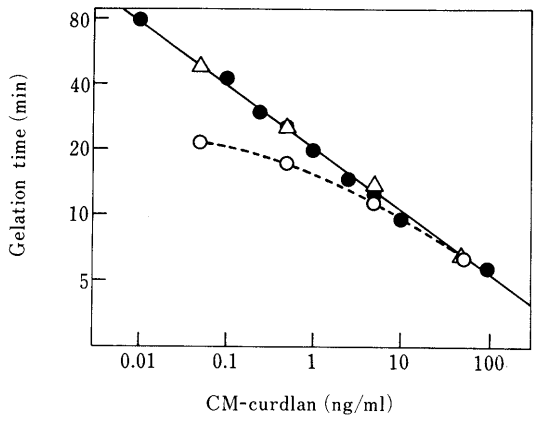

Fig. 7. Effect of Polymyxin B on Dose–Response Curves of CM-Curdlan

●, CM-curdlan only; ○, CM-curdlan containing 0.125 EU/ml endotoxin; △, CM-curdlan containing 0.125 EU/ml endotoxin with 0.2 mg/ml polymyxin B. The solid line in the figure is the regression line of CM-curdlan.

Discussion

The main purpose of this study was to develop a simple method for discriminating between endotoxin and (1→3)-β-D-glucan using the turbidimetric kinetic method and ordinary LAL reagents. We analyzed the difference of the courses of the reactions between endotoxin and LAL and between (1→3)-β-D-glucan and LAL on the Toxinometer ET-201. As reported previously,[7] the reaction course of LAL with (1→3)-β-D-glucan was quite different from that of LAL with endotoxin. Zhang *et al.*[9] have reported that (1→3)-β-D-glucan seems to behave in an intrinsically different way from endotoxin in an enzyme-linked immunosorbent assay with a monoclonal antibody against the coagulogen. They pointed out that (1→3)-β-D-glucan is distinguishable by its much slower rate of reaction. Our observations made with the turbidimetric kinetic method agreed with their report. Additionally, we found that the transmittance ratio of the reaction between (1→3)-β-D-glucan and LAL was reduced earlier than in the reaction between endotoxin and LAL.

The T_p–D_{max} plot distinguished (1→3)-β-D-glucan and endotoxin. Their mixture was plotted at a particular position corresponding to their respective concentrations. This indicated the possibility of their simultaneous assay by construction of a calibration lattice for them. However, the construction of the calibration lattice seemed to complex to analyze statistically.

Curdlan is a naturally occurring (1→3)-β-D-glucan, and is insoluble in water. CM-curdlan is an artificial derivative of (1→3)-β-D-glucan, and is soluble in water. Because the reactivities of curdlan and CM-curdlan with LAL were similar, both substances could be used for this study.

The addition of (1→3)-β-D-glucan to higher concentrations of endotoxin shortened T_p, but had little effect on D_{max}. Correlation between endotoxin concentration and D_{max} was good. Therefore, a quantitative assay of endotoxin without the effect of (1→3)-β-D-glucan was thought possible using the ET–D_{max} plot. As shown in Fig. 5, 10 ng/ml curdlan did not affect the D_{max} at 0.2 EU/ml endotoxin. This indicates that this plot is useful for the bacterial endotoxins test at a common endotoxin limit of 0.25 EU/ml, the limit for WFI required by the United States and the Japanese

government. Actually, spiked endotoxin (0.125 EU/ml, half of the limit for WFI) in 5 ng/ml or less CM-curdlan solution was recovered well (between 89.6 and 112.8%) by calculation from D_{max}. On the other hand, spiked endotoxin in 0.05 ng/ml or more CM-curdlan was recovered excessively (136.0% or more) by calculation from T_g. The ET–D_{max} plot is thus useful for the measurement of endotoxin when samples may be contaminated with a small amount of (1→3)-β-D-glucan.

Polymyxin B combines specifically with lipopolysaccharide (LPS)[10]; the results shown in Fig. 6 confirmed this. Because the addition of more than 0.2 mg/ml polymyxin B to the reaction mixture caused a non-specific turbidity change that influenced the assay with the Toxinometer (data not shown), only this quantity of 0.2 mg/ml polymyxin B was used in this study. (1→3)-β-D-Glucan was measured without the influence of 0.125 EU/ml endotoxin by the turbidimetric kinetic method in the presence of 0.2 mg/ml polymyxin B. Although this addition did not completely inhibit the potency of 0.2 EU/ml endotoxin, we clearly recognized a decrease in endotoxin reactivity with LAL. Therefore, the LAL containing polymyxin B is useful not only for measurement of (1→3)-β-D-glucan contaminated by a small amount of endotoxin but also for confirmation of whether a sample contains only (1→3)-β-D-glucan or contains both (1→3)-β-D-glucan and endotoxin.

This study aimed to provide a method for discriminating between endotoxin and (1→3)-β-D-glucan in a LAL test with the Toxinometer. We established two plots, the T_p–D_{max} plot and the ET–D_{max} plot, the former showing whether endotoxin or (1→3)-β-D-glucan is the main substance activating LAL. When endotoxin is the main activator of LAL, the ET–D_{max} plot will provide the best measurement. When (1→3)-β-D-glucan is the main activator, the addition of polymyxin B to the reaction mixture will allow better measurement. Consequently, we propose the following procedure as a LAL test to discriminate between endotoxin and (1→3)-β-D-glucan.

(1) Identify the main substance (endotoxin or (1→3)-β-D-glucan) triggering the activation of LAL using the T_p–D_{max} plot.

(2) Use the appropriate method to measure the main substance: the ET–D_{max} plot for endotoxin or the LAL with polymyxin B for (1→3)-β-D-glucan.

Former data on the Toxinometer can be also analyzed by this method. The method does not require any special reagent and may enhance the utility of the turbidimetric kinetic LAL test.

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