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Endotoxin-Inactivating Activity in Normal and Pathological Human Blood Samples

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The endotoxin-specific chromogenic test revealed that plasma endotoxin-inactivating activity was markedly diminished by endotoxemia, but not by fungemia or by dialysis with cellulose membranes, suggesting that fungal polysaccharides and other nonendotoxic, *Limulus*-reactive materials do not consume endotoxin-inactivating factors in the blood. There was a close negative correlation between plasma endotoxin concentration and endotoxin-inactivating activity. The specificity of the test was improved by fractionating amebocyte lysate and using only the factors that constitute the endotoxin-sensitive coagulation pathway of the horseshoe crab. This test was able to differentiate endotoxemia from fungemia and from contamination with other nonendotoxic, *Limulus*-reactive materials.

Despite the presence of a large amount of endotoxin in the intestinal lumen, little is detected in normal human blood. This low concentration seems to be maintained through several mechanisms operating at various levels. The intestinal wall barrier apparently prevents most of the endotoxin from gaining access to the bloodstream. That which seeps into the portal vein is detoxified by the reticuloendothelial system or by the blood itself (13). Phagocytes are primarily involved in this detoxification in the reticuloendothelial system (6), whereas various serum components are thought to be responsible for the inactivation of endotoxin in the blood (3–5, 12, 14, 15), although the nature of the endotoxin-inactivating factor (EIF) in serum remains controversial.

A precise method for measuring endotoxin-inactivating activity is a prerequisite for advancing the study of such serum factors. The conventional *Limulus* test, whether by gelation or by colorimetry, is not suitable for this purpose since it is not specific for endotoxin because of the presence of a (1→3)-β-D-glucan-sensitive factor G (7) in the *Limulus* amebocyte lysate besides endotoxin-sensitive factor C (8) (Fig. 1-I). In fact natural lysate responds directly to fungal polysaccharides and other nonendotoxic, *Limulus*-reactive materials as well through the mediation of factor G (2, 10).

In this study, we applied our new development, an endotoxin-specific chromogenic test (10), to the measurement of endotoxin-inactivating activity in normal and pathological human blood samples.

MATERIALS AND METHODS

Glassware. All glassware was dry-heated to 250°C for 2 h and checked for contamination with endotoxin or other *Limulus*-positive materials by a conventional chromogenic *Limulus* test (Toxicolor Test; Seikagaku Kogyo, Tokyo, Japan) (9).

Blood samples. Platelet-rich plasma (PRP) was prepared by centrifuging heparinized human venous blood at 150 × *g* for 10 min and platelet-poor plasma (PPP) at 1,000 × *g* for 10 min; serum was obtained by centrifugation at 1,000 × *g* for 10 min without an anticoagulant. Centrifugation was done at 4°C, and all blood samples were placed in an ice bath. Clinical samples were held in a freezer at −80°C when not assayed immediately.

Pretreatment of blood samples for endotoxin assay. *Limulus* test-interfering factors were removed by exposing 0.1 ml of plasma or serum to 0.2 ml of 0.32 M perchloric acid (PCA) at 37°C for 20 min. The supernatant obtained by centrifugation at 3,000 rpm for 15 min was used for assay after being neutralized with an equal volume of 0.18 M NaOH (9).

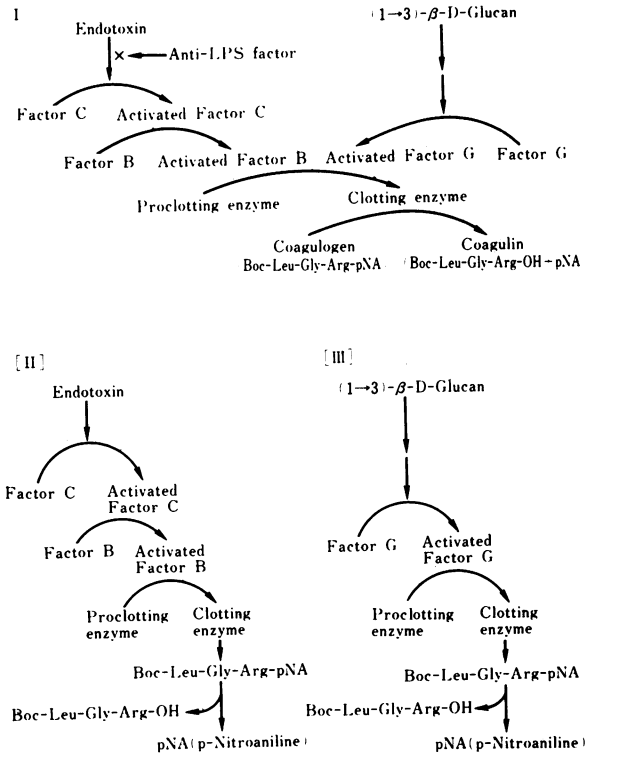

FIG. 1. Outline of the *Limulus* blood coagulation cascade and the principles of the usual chromogenic *Limulus* test (I), the ES test (II), and the G test (III). LPS, Lipopolysaccharide.

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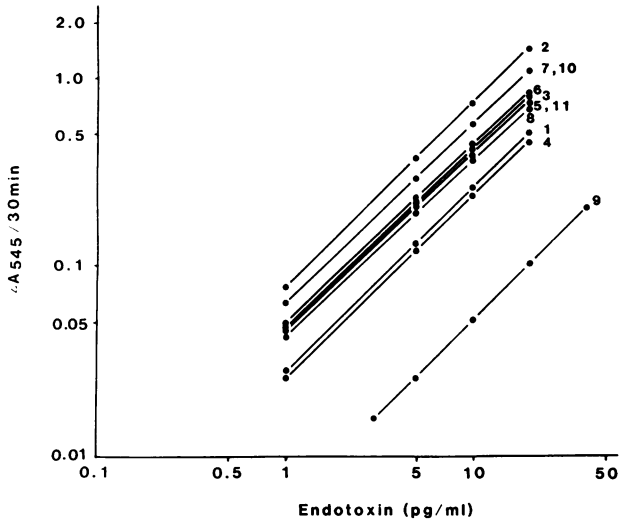

FIG. 2. Regression lines of the ES test against 11 different endotoxins: 1, *E. coli* O111:B4; 2, *E. coli* O55:B5; 3, *Shigella flexneri*; 4, *Serratia marcescens*; 5, *Salmonella typhosa*; 6, *Salmonella enteritidis*; 7, *Salmonella abortus equi*; 8, *Salmonella typhimurium*; 9, *P. aeruginosa*; 10, Novo-Pyrexal NP1; 11, USP reference standard.

Endotoxins. The following 11 Westphal-type endotoxins were used: *Escherichia coli* O111:B4, *E. coli* O55:B5, and *Shigella flexneri* endotoxins (Difco Laboratories, Detroit, Mich.); *Serratia marcescens*, *Salmonella typhosa*, *Salmonella enteritidis*, *Salmonella abortus equi*, and *S. typhimurium* endotoxins (Sigma Chemical Co., St. Louis, Mo.); *Pseudomonas aeruginosa* endotoxin (List Biological Laboratories, Campbell, Calif.); USP reference standard endotoxin (*E. coli* O113) (U.S. Pharmacopeial Convention); and Novo-Pyrexal endotoxin standard NP1 (*Salmonella abortus equi*) (Pyroquant Diagnostik GmbH, Walldorf, FRG).

Glucan. (1→3)-β-D-Glucan from *Alcaligenes faecalis* var. *myxogenes* IFO13140 (Curdlan; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used.

Endotoxin assay. An endotoxin-specific chromogenic test (ES test; Seikagaku Kogyo) was used (10). This test consists of factor G-free *Limulus*-amebocyte lysate and a chromogenic substrate, *t*-butyloxycarbonyl (Boc)-Leu-Gly-Arg-*p*-nitroanilide (pNA). A sample (0.1 ml) was added to a 0.1-ml portion of ES test dissolved in 0.2 M Tris hydrochloride buffer (pH 8.0), and the mixture was incubated at 37°C for 30 min. Absorbance was measured at 545 nm after diazotization. The standard curve was plotted by using *E. coli* O111:B4 endotoxin in distilled water. A conventional chromogenic *Limulus* test (Toxicolor test) was also used for comparison (9).

Assay of factor G-reactive material. Equal volumes of a factor G fraction and a fraction containing proclotting enzyme were combined with Boc-Leu-Gly-Arg-pNA (0.4 mM final concentration) and then lyophilized (G test) (10). A sample (0.1 ml) was added to a 0.1-ml portion of G test dissolved in 0.2 M Tris hydrochloride buffer (pH 8.0). Subsequent procedures were the same as for the ES test. The standard curve was plotted by using (1→3)-β-D-glucan. The stock solution of (1→3)-β-D-glucan (1.0 μg/ml) was prepared by dissolving it in 0.05 M NaOH, and the dilutions were made with 0.01 M NaOH.

Comparison of regression lines of ES test against 11 different endotoxins. To ensure that the ES test was applicable to different endotoxins, serial dilutions of 11 endotoxins in distilled water were assayed with it, and their regression lines were compared on a bilogarithmic scale. One USP reference standard endotoxin unit was calculated as 0.2 ng (1).

Recovery of endotoxin added to normal plasma and serum measured by the ES test coupled with the PCA pretreatment. To show that the ES test was applicable to blood samples when coupled with PCA treatment, nine different endotoxins were added separately to plasma and serum, and their

TABLE 1. Recovery of endotoxins from PRP, PPP, and serum with ES test combined with PCA treatment

Endotoxin	% Recovery (mean ± SD, n = 6)		
	PRP	PPP	Serum
E. coli O111:B4	98.2 ± 1.9	96.7 ± 1.9	98.8 ± 2.2
E. coli O55:B5	93.4 ± 2.5	94.5 ± 3.0	94.6 ± 3.9
Shigella flexneri	99.3 ± 2.5	98.8 ± 3.1	99.7 ± 2.9
Serratia marcescens	100.4 ± 2.7	100.7 ± 2.4	99.9 ± 1.9
Salmonella typhosa	99.5 ± 1.4	99.4 ± 1.6	101.0 ± 3.5
Salmonella enteritidis	98.7 ± 1.0	97.1 ± 2.5	100.9 ± 2.7
Salmonella abortus equi	96.5 ± 4.0	96.2 ± 3.2	97.7 ± 3.5
Salmonella typhimurium	102.1 ± 3.8	99.7 ± 2.7	100.2 ± 2.8
P. aeruginosa	98.0 ± 1.7	101.5 ± 3.8	101.6 ± 4.0

TABLE 2. Endotoxin-inactivating activity and test results[a]

PRP sample[b]	Concn (pg/ml)[c]			CD_{50} by ES test (pg/ml per 2 min)
	Toxicolor test	ES test	G test	
Normal	2.2 ± 1.5	0.8 ± 0.6	0.2 ± 0.3	2.7 (± 0.6) × 10^7
Endotoxemia				
a	255.9	87.8	1.4	3.4 × 10^2
b	88.5	86.9	0.6	3.7 × 10^2
c	162.8	68.6	1.2	6.4 × 10^2
d	75.5	61.2	0.2	7.5 × 10^2
e	119.8	59.7	0.8	7.8 × 10^2
f	112.6	57.2	0.9	8.0 × 10^2
g	46.9	40.1	0.2	10.6 × 10^2
Fungemia				
h	68.6	0.3	268.9	2.6 × 10^7
i	395.9	0.0	1,733.1	3.2 × 10^7
j	103.0	1.2	328.7	2.8 × 10^7
k	308.8	0.0	1,719.0	3.0 × 10^7
l	52.9	2.5	167.3	2.2 × 10^7
Hemodialyzed blood				
m	1,107.7	2.2	4,628.0	3.0 × 10^7
n	685.2	1.6	2,753.6	2.6 × 10^7
o	1,198.4	2.5	4,635.5	2.8 × 10^7
p	552.4	1.8	2,052.1	2.8 × 10^7
q	1.080.1	1.2	4,104.5	2.9 × 10^7
r	1,275.3	2.5	5,321.4	2.8 × 10^7

[a] *E. coli* O111:B4 endotoxin was used as a reference in the Toxicolor and ES tests and (1 → 3)-β-D-glucan as a reference in the G test.

[b] Blood cultures were positive for *P. aeruginosa* (e). *C. guilliermondii* (j). and *C. albicans* (k). Samples h and j were from the same case; sample i was from a case of pulmonary aspergillosis; and sample l was from an extensive burn infected with *C. albicans*. Values for normal samples (n = 20) are means ± standard deviations.

[c] Toxicolor test measures *Limulus*-reactive material, ES test measures endotoxin, and G test measures factor G-reactive material.

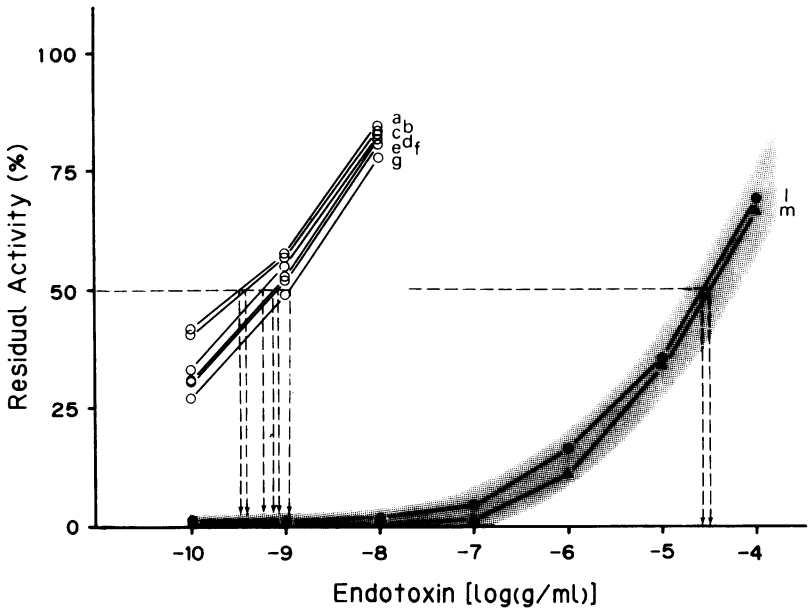

FIG. 3. Endotoxin-inactivating activity (PRP) in endotoxemia (○), in fungemia (●), and in blood dialyzed with cellulose membranes (▲). Shaded area indicates normal range. Dashed lines indicate CD_{50} readings for each case. Lowercase letters representing samples correspond to those in Table 2.

recovery was examined. After 0.01 ml of an endotoxin solution (1.0 ng/ml) was added to 0.1 ml of six normal plasma and serum samples in an ice bath, 0.2 ml of 0.32 M PCA was added, and the mixture was incubated at 37°C for 20 min. Denatured material was removed by centrifugation at 3,000 rpm for 15 min, and the supernatant (0.05 ml) was neutralized with an equal volume of 0.18 M NaOH and subjected to the ES test.

Determination of endotoxin-inactivating activity in normal plasma and serum. *E. coli* O111:B4 endotoxin (0.01 ml) was added to 0.1 ml of 20 normal plasma and serum samples in an ice bath in final concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} g/ml. The mixtures were incubated at 37°C for 2 min, and the residual endotoxin was assayed by the ES test after PCA treatment. Recovery was expressed as the percentage of absorbance of a sample measured without the 2-min preincubation. The rate of endotoxin inactivation at which 50% of the added endotoxin was inactivated with 1 ml of plasma or serum in 2 min was referred to as the 50% endotoxin clearance dose of EIF at 37°C (CD_{50}).

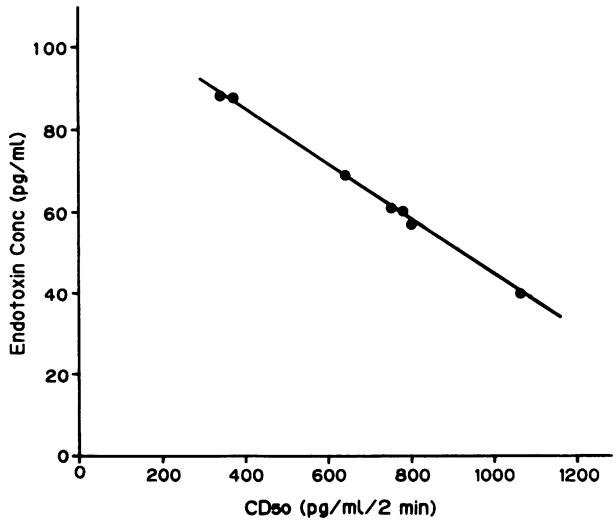

FIG. 4. Negative correlation of endotoxin concentration with CD_{50} in endotoxemia.

Endotoxin-inactivating activity in pathological states. The CD_{50} was measured as described above in six cases (seven samples) of endotoxemia, four cases (five samples) of systemic fungal infection, and six cases of chronic renal failure under hemodialysis with cellulose membranes. Diagnosis of endotoxemia was based on the endotoxin level as determined by the ES test (normal, less than 3 pg of endotoxin per ml) (10). More than 3 pg/ml by the ES test was regarded as endotoxemia. One of the six cases yielded a blood culture positive for *Pseudomonas aeruginosa*. Of the four fungal infections, two yielded a blood culture positive for fungi, *Candida albicans* and *C. guilliermondii*. The remaining two cases were an acute myelomonocytic leukemia complicated by pulmonary aspergillosis which was confirmed at autopsy and an extensive burn infected with *C. albicans*.

RESULTS

Regression lines of the ES test against 11 different endotoxins. The ES test yielded good linearity between endotoxin concentration and absorbance for all 11 endotoxins examined, all regression lines being parallel to one another (Fig. 2).

Recovery test. With PCA pretreatment, the ES test yielded nearly 100% recovery from normal PRP, PPP, and serum for each of the nine different endotoxins (Table 1).

Endotoxin-inactivating activity of normal plasma and serum. The CD_{50} of EIF in normal PRP, PPP, and serum was 26.6 ± 6.2, 32.4 ± 7.4, and 40.3 ± 9.2 μg/ml per 2 min (mean ± standard deviation, n = 20), respectively.

Endotoxin-inactivating activity in pathological states. There was about a 10^5-fold decrease in CD_{50} in endotoxemia,

whereas in systemic fungal infections and in blood samples dialyzed with cellulose membranes it remained within normal limits (Table 2 and Fig. 3). In endotoxemia, both the Toxicolor and ES tests yielded high values, with negligible reaction to the G test; in fungal infections and dialysis, the Toxicolor and G tests showed high values, with almost no reaction to the ES test (Table 2). A close negative correlation was noted between plasma endotoxin concentration and CD_{50} in endotoxemia (Fig. 4).

DISCUSSION

Because of its high sensitivity and quantitative measurement, a colorimetric method with chromogenic substrate for determining endotoxin has been replacing the original *Limulus* gelation test for the past several years. Recently, however, the *Limulus* test has proved to be nonspecific for endotoxin; in fact, it reacts with a trace amount of (1→3)-β-D-glucan (7), fungal polysaccharides (10), and rinses of a cellulose hemodialyzer (11) as well. Fortunately, the latter substances activate *Limulus* coagulation enzymes via a pathway different from that of endotoxin. Thus, we fractionated amebocyte lysate from *Tachypleus tridentatus*, a Japanese horseshoe crab, by the method of Iwanaga et al. (2) to separate the two pathways and developed an endotoxin-specific assay (ES test, Fig. 1-II) and a glucan assay (G test, Fig. 1-III) (10).

As shown in Table 2, a conventional *Limulus* test, because of the use of whole lysate, does not differentiate endotoxemia from fungemia or contamination with other factor G-reactive materials; the ES test, on the other hand, clearly differentiates endotoxemia from the latter conditions. The parallel regression lines against different endotoxins indicate the reliability of the ES test in measuring an unknown type of endotoxin in samples; the full recovery of various endotoxins added to blood samples illustrates the applicability of the PCA pretreatment to the ES test in eliminating *Limulus* test-interfering factors in a blood sample.

With this entirely new test, we measured endotoxin-inactivating activity in normal, endotoxemic, fungemic, and dialyzed blood. The CD_{50}s in normal PRP, PPP, and serum were approximately equal to the values found by a conventional chromogenic *Limulus* test (9). The advantage of the ES test is that it allows determination of the inactivating activity even in blood contaminated with fungal or other nonendotoxic, *Limulus*-reactive materials, since these substances do not interfere with this test as they do with the conventional test. In endotoxemia, the CD_{50} was decreased to as low as 10^{-5} times the normal, with a close negative correlation with plasma endotoxin concentration. Thus, the ES test will be helpful not only in the confirmatory diagnosis of endotoxemia, but also in the study of endotoxin inactivation mechanisms. Normal CD_{50} in fungemia and in dialyzed blood with cellulose membranes suggests that EIFs are not consumed by fungal or dialyzer-derived polysaccharides. This may be due to the lack of lipid components in these polysaccharides, since EIF is likely to bind to or degrade a lipid portion of endotoxin (lipopolysaccharide), lipid A, which is responsible for the activation of factor C. In view of its close inverse relationship with plasma endotoxin level and no consumption by fungal polysaccharides or dialyzer-derived materials, EIF seems to be highly selective for endotoxin.

The *Limulus*-reactive materials in hemodialyzed blood appear to be a glucanlike substance that contaminates cellulose, because rinses from both a cellulose-based dialyzer and its stock powder of cellulose react with the G test (unpublished data) and because cellulose itself is not reactive to amebocyte lysate. This contaminant may account for a high incidence of false-positive endotoxemia results for hemodialyzed patients reported in earlier papers. Since the influx of such foreign material for an extended period is not favorable even if it is not pyrogenic, parenteral drugs, dialyzers, and other medical devices that will be in direct contact with circulating blood should be scrutinized not only by the pyrogen test with rabbits but by the *Limulus* test as well.

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