Plasma (1→3)- β -D-Glucan Assay and Immunohistochemical Staining of (1→3)- β -D-Glucan in the Fungal Cell Walls Using a Novel Horseshoe Crab Protein (T-GBP) That Specifically Binds to (1→3)- β -D-Glucan

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A highly sensitive enzyme-linked immunosorbent assay specific to (1→3)- β -D-glucans (GBP-ELISA) has been developed using a novel (1→3)- β -D-glucan-binding protein (T-GBP), which was purified from the amebocyte lysate of the Japanese horseshoe crab, *Tachypleus tridentatus*. This method allowed quantitation of the glucans in a concentration range of 0.1–1,000 ng/ml, regardless of linear and branched structures,

and was applied to determine the amounts of (1→3)- β -D-glucan in human and animal plasmas for diagnosis of fungemia. High levels of plasma glucan contents in clinical samples were found to be correlated closely with the severity of fungal infection. T-GBP was successfully utilized for indirect immunofluorescence staining of (1→3)- β -D-glucan in *Candida albicans* cell walls. J. Clin. Lab. Anal. 11:104–109. © 1997 Wiley-Liss, Inc.

Key words: limulus amebocyte lysate; chromogenic substrate; factor G; (1→3)- β -D-glucan-binding protein (T-GBP); ELISA

INTRODUCTION

In 1968, Levin and Bang originated a limulus gelation test for detecting endotoxin on the basis of their discovery that a minute amount of endotoxin induces coagulation of *Limulus* amebocyte lysate in vitro (1). The test involving the coagulation cascade has developed a highly sensitive chromogenic assay for endotoxin and is now widely used for clinical as well as research purposes (2).

Meanwhile, it has become evident that the amebocyte lysate of Japanese horseshoe crab, *Tachypleus tridentatus*, contains a protease zymogen that is activated with (1→3)- β -D-glucan, a major fungal cell wall constituent, resulting in the operation of the coagulation system. The responsible proenzyme, factor G, was partially purified, characterized, and allowed its application in the diagnosis of deep mycosis by detecting (1→3)- β -D-glucan in plasma (3–6).

Recently we have found in *Tachypleus* amebocyte lysate a novel protein that specifically binds to (1→3)- β -D-glucan, thereby inhibiting the activation of factor G by the glucan. The protein was named *Tachypleus* (1→3)- β -D-glucan-binding protein (T-GBP) and its physicochemical properties have been partially elucidated (7).

In the present work, we report the application of T-GBP to enzyme-linked immunosorbent assay for plasma (1→3)- β -D-glucan and to immunohistochemical staining of fungal cell walls.

MATERIALS AND METHODS

Glassware was heated to 260°C for 2 hours to make it (1→3)- β -D-glucan- as well as endotoxin-free. Instruments that cannot stand dry heating were autoclaved at 121°C for 20–120 min. Reagents were decontaminated by adding, in a ratio of 1g/50 ml, of activated charcoal (Sigma Chemical Co., St. Louis, MO), which had been washed with phosphoric and sulfuric acids and then passed through Millex-GS (0.22 μ m; Millipore Corp., Bedford, MA). Decontamination was checked with two *Tachypleus* chromogenic limulus reagents (Seikagaku Corporation, Tokyo): one is specific to (1→3)- β -D-glucan (Gluspecy), the other to endotoxin (Endospecy), respectively.

Pachyman, a linear (1→3)- β -D-glucan was prepared from *Poria cocos* mycelia according to the method of Saito et al.

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(8), dissolved in 0.1 mol/l NaOH, and used as a standard after dilution with 0.01 mol/l NaOH.

Linear (1→3)-β-D-glucans used were curdian (Wako Chemical Industries, Osaka), paramylon (a gift of Prof. B.A. Stone at Melbourne University), and carboxymethylated curdian (degree of substitution, 0.63), which was prepared according to the method of Clarke and Stone (9).

The following (1→6), (1→3)-β-D-glucans were used; *Laminaria digitata* laminarin (Sigma Chemical Co.), schizophyllan (Soniphyllan; Kaken Chemical Co., Tokyo), and lentinan (Ajinomoto Co., Tokyo). Barley β-D-glucan (Sigma Chemical Co.), a (1→4), (1→3)-β-D-glucan was also examined.

Related D-glucans used were as follows; cellulose, (1→4)-β-D-glucan (Nakarai Tesque, Kyoto), yeast α-D-mannan (Sigma Chemical Co.), *Leuconostoc* dextran, a (1→6)-α-D-glucan (Seikagaku Corp., Tokyo), and *Pullularia pullulans* pullulan, a (1→6), (1→4)-α-D-glucan (Hayashibara Biochemical, Okayama).

A peptidoglycan was purified from *Micrococcus luteus* powder by the method of Heilmann (10). *Escherichia coli* 0111:B4 and *Salmonella minnesota* lipopolysaccharide (LPS) were obtained from List Biological Laboratories (CA). These bacterial glycans were tested for their reactivity to T-GBP as mentioned later.

T-GBP was purified from the amebocyte lysate (total volume, 900 ml; protein content, 5 mg/ml), which were aseptically obtained from hemolymph tapped from *T. tridentatus* hearts, as described previously (7).

Assay of T-GBP Activity

Samples containing T-GBP and pachyman (2.5 pg) were mixed and incubated at 37°C for 10 min as described elsewhere (7). The amounts of (1→3)-β-D-glucan were determined with Gluspecy and compared with the value obtained without added T-GBP. One unit of the activity was defined as the amount of T-GBP capable of causing 50% inhibition of the activation of factor G under the standard assay condition.

Biotination of T-GBP

T-GBP was biotininated according to the method of Bayer and Wilchek (11). Purified T-GBP (1.1 mg) was dissolved in 0.5 ml of a buffer (pH 7.0) comprising *N,N*-dimethylformamide that contained 0.01 mol/l HCl and 0.05 mol/l KH₂PO₄, and incubated with *N*-hydroxysuccinimido-biotin (13.0 mg) in the buffer (6 ml) at 20°C. After 5 hours, the reaction was stopped by dropwise addition of glacial acetic acid to lower the pH of the mixture to 3.0. The biotininated T-GBP was desalted by gel permeation chromatography (g.p.c.) on a Cellulofine GH-20-m column (2.2 × 95 cm, Seikagaku Corp.), applied to a CM-Sepharose CL-6B column (2.5 × 45 cm) pre-equilibrated with 0.01 mol/l ammonium acetate buffer (pH 4.5) and then eluted with a linear gradient of NaCl (0–

1.0 mol/l) in the buffer (total volume, 200 ml). Fraction (1 ml) were collected and monitored for the inhibitory activity and at 280 nm. The active fractions were pooled, desalted, and stored at –80°C. A yield of pure biotininated T-GBP was 650 μg.

Preparation of Rabbit Anti-T-GBP Antibody

Purified T-GBP (200 μg each) was mixed with Freund's complete adjuvant and injected subcutaneously in the backs of two male rabbits (JW, 1.8 kg) four times every 2 weeks, followed in another 2 weeks by a booster injection (100 μg each). One week later, the rabbits were bled and then antisera (96 ml) were collected. The titers of the antisera were 1:64 by a double diffusion method. The antisera were brought to 16.5% (w/v) saturation by addition of saturated ammonium sulfate, and the resulting precipitate were collected by centrifugation. The immunoglobulins (IgGs) in the precipitate were washed with 16.5% (w/v) ammonium sulfate, dissolved in 0.1 mol/l Tris-HCl buffer (pH 8.0), and desalted by g.p.c. on a Cellulofine GH-25-m column (2.2 × 95 cm) as above. Chromatography on a protein A-Sepharose 4B column (2.5 × 20 cm, Pharmacia Biotech, Uppsala) pre-equilibrated with 0.01 mol/l phosphate buffer (pH 7.0) containing 0.15 mol/l NaCl effected further purification of the antibody. Fractions containing the IgGs were percolated through a syringe filter (0.22 μm, Millex). Purified IgGs (total amount, 45 mg in 4.5 ml) thus obtained were stored at 4°C.

Enzyme-linked Immunosorbent Assay for (1→3)-β-D-Glucan (GBP-ELISA)

Fifty microliters of mouse monoclonal anti-(1→3)-β-D-glucan IgG-κ antibody (5 μg/ml in 0.05 mol/l sodium bicarbonate buffer, pH 9.6; Biosupplies Australia, Parkville Victoria, Australia), which recognized consecutive alignments of (1→3)-β-D-glucopyranosyl residues (12), were dispensed into each well in a Toxipet Plate 96F [(1→3)-β-D-glucan- and endotoxin-free, Seikagaku Corp.] and left standing at 4°C for 12 hours. The wells were washed three times with phosphate-buffered saline (PBS) and blocked by incubation with 2% (w/v) human serum albumin at 37°C for 90 min. After washing three times with PBS containing 0.1% (w/v) Tween-20, 50 μl of varying concentrations (0.1, 1, 10, 100, 1,000 ng/ml) of pachyman were added to the wells, incubated at 37°C for 120 min, and washed three times with PBS containing 0.1% (w/v) Tween-20. Then, 50 μl of biotininated T-GBP (2.0 μg/ml) was added to each well, incubated at 37°C for 120 min, and washed three times with PBS containing 0.1% (w/v). After addition of 1,000 times-diluted horseradish peroxidase-conjugated streptavidin (100 μl) to each well, the plate was incubated and washed as above. One hundred microliters of a substrate mixture (total volume, 10 ml) consisting of 10 mg of tetramethyl-benzidine and 2 μl of 30% (w/v) H₂O₂ in 0.05 mol/l acetate buffer (pH 5.0) were added to each well and left at room temperature for 15 min. The reaction was stopped

by adding 50 μ l of 2 mol/l sulfuric acid. Absorbance at 450 nm was measured with a Wellreader SK601 (Seikagaku Corp).

Reactivity of GBP-ELISA

Pachyman, curdlan, CM-curdlan, paramylon, laminarin, schizophyllan, lentinan, barley β -D-glucan, cellulose, yeast α -D-mannan, dextran, pullulan, *E. coli* 0111:B4, and *S. minnesota* LPSs, and a *M. luteus* peptidoglycan (5 ng/ml in all) were tested for the reactivity by GBP-ELISA.

Recoveries of (1 $\rightarrow$ 3)- β -D-Glucan Added to Plasma

Aliquot (10 μ l) of three different concentrations of pachyman (10, 100, 1,000 ng/ml) as the standard were added to 100 μ l of plasma from human adults (male) and mammals including mice (ICR, male), rats (Wister, male), and guinea pigs (Hartley, male) in an ice bath. A total of 200 μ l of 0.32 mol/l perchloric acid were added to each sample, and the mixture were incubated at 37°C for 20 min (2). After centrifugation, the supernatant was neutralized with an equal amount of 0.18 mol/l NaOH prior to GBP-ELISA. Normal saline was used as the control.

In Vivo Clearance of (1 $\rightarrow$ 3)- β -D-Glucan in Mice Infected With *Candida tropicalis*

Prednisolone (100 μ g/g body weight) was injected into three mice (ICR, 6w, male), and after 3 days, a cell suspension (100 μ l, 10^6 /ml) of *Candida tropicalis* (AM-CT101), which was maintained at the Department of Microbiology, Azabu University, grown on Sabouraud glucose agar was injected through a tail vein. Blood was drawn every day for 17 days, using heparin as an anticoagulant. Three mice were treated with amphotericin B (2 μ g/g of body weight) from 4th day. (1 $\rightarrow$ 3)- β -D-glucan concentrations in plasma were determined by GBP-ELISA as described above. In addition, viable yeasts were counted on the kidneys and livers and expressed as colony-forming units/g of tissue (cfu/g).

Clinical Evaluation

GBP-ELISA was applied to examine 25 stocked clinical plasma samples, which include 10 healthy volunteers (7 male, 3 female; median age 32, range 24–43 years), 9 deep fungal infections [6 with microbiologically documented fungemia and 3 with necropsy-verified deep mycosis (5 candidiasis, 2 aspergillus pneumonia, 2 cryptococcosis)], and 6 other bacteremia (4 with gram-negative bacteremia and 2 with gram-positive bacteremia).

Immunohistochemical Staining of (1 $\rightarrow$ 3)- β -D-Glucan Localized in Fungal Cell Wall

Yeast cells of *C. albicans* (IFO 1385) cultured in a Yeast Nitrogen Base (Difco Laboratories, MI) containing 0.5% (w/v)

glucose at 37°C for 48 h was washed three times with PBS, and 20 μ l of the cell suspension was fixed on a slide glass over flame. Twenty μ l of T-GBP (10 μ g/ml) was added to the smear and incubated at 37°C for 60 min. After washing three times with PBS, 20 μ l of anti-T-GBP antibody (a 100-fold diluted solution of the stock solution) was added, incubated at 37°C for 60 min, and then washed three times with PBS. Fluorescent staining was performed by addition of 20 μ l of FITC-protein A (50 μ g/ml) followed by incubation and washing with PBS as above. Yeast cells in the smears were observed with a Olympus AH-2-FL fluorescent microscope in an excitation wavelength at 490 nm and micrographs were taken on a Kodak Ektachrome film (Eastman-Kodak Co., Rochester, NY). T-GBP added was replaced with PBS in a negative control.

RESULTS

As seen in Figure 1, a good linear relationship was noted between the absorbance at 450 nm and the concentrations of pachyman (0.1–1,000 ng/ml) as a standard.

Specificity of GBP-ELISA

GBP-ELISA was found to react specifically with (1 $\rightarrow$ 3)- β -D-glucans to the same extent, regardless of linear and branched types. Laminarans with degrees of polymerization of 20–25 were also reactive. On the contrary, no measurable reaction was observed with other glycans, e.g., yeast α -mannan, two LPSs, and a *M. luteus* peptidoglycan (Table 1).

Addition Recovery Test

Recoveries of pachyman as a standard from plasmas of various animal species (%n = 3 in each) were as follows:

Fig. 1. A calibration curve of GBP-ELISA using pachyman as the standard (1 $\rightarrow$ 3)- β -D-glucan.

TABLE 1. Reactivity of GBP-ELISA to Various (1→3)-β-D-Glucans

	Type of linkages	Mn ^a	Δ450 nm ^b
Pachyman	(1→3)-β-D-	80000	0.235
Curdlan	(1→3)-β-D-	>136000	0.254
CM-curdlan	(1→3)-β-D-	> 95000	0.279
Paramylon	(1→3)-β-D-	>118000	0.191
Laminarin	(1→6)-(1→3)-β-D-	5850	0.272
(<i>L. digitata</i>)			
Schizophyllan	(1→6),(1→3)-β-D-	76800	0.139
(Soniphyllan)			
Lentinan	(1→6),(1→3)-β-D-	94700	0.147
Barley β-D-glucan	(1→4),(1→3)-β-D-	23100	0.171
Cellulose	(1→4)-β-D-	—	0.010
Yeast α-D-mannan	(1→2),(1→3), (1→6)-α-D-	—	0.015
Dextran	(1→6)-α-D-	—	0.004
Pullulan	(1→4),(1→6)-α-D-	—	0.006
Lipopolysaccharide			
(<i>E. coli</i> 0111:B4)	—	—	0.005
(<i>S. minnesota</i>)	—	—	0.005
Peptidoglycan	—	—	0.007
(<i>M. luteus</i>)			

^aNumber-average molecular weight^bAbsorbance at 450 nm obtained with each sample, that of the control.

96.5 ± 7.5 (human), 88.9 ± 6.6 (mouse), 98.1 ± 9.0 (rat), 92.0 ± 5.2 (guinea pig), and 81.6 ± 4.3 (rabbit).

Clearance of (1→3)-β-D-Glucan in Mice Plasma Infected with *Candida tropicalis*

Figure 2 shows the changes in the mean values of plasma concentrations of (1→3)-β-D-glucan in three mice, to whom *C. tropicalis* cells (10⁶/ml) were intravenously injected. A peak (a mean value = 2,830 pg/ml at the maxima) was reached 2 days after infusion, decreased gradually and turned out to be undetectable on 17th day. Obviously a good correlation was noted between the numbers of viable yeast cells in the kidneys and concentrations of the plasma glucan. However, such

Fig. 2. Clearance of plasma (1→3)-β-D-glucan in mice after injection with *Candida tropicalis* cells. The amounts of plasma (1→3)-β-D-glucan were determined by GBP-ELISA.

was not the case with the livers (data not shown). A remarkable decrease in the numbers of viable yeast cells was noticed in the kidneys of three amphotericin B-injected mice, and the glucan in their plasma diminished rapidly and disappeared on 5th day.

Clinical Application

Table 2 summarized the values of the glucan concentrations in the plasmas of patients suffering from gram-negative and gram-positive bacteremia as well as in those of healthy individuals. No measurable amount (< 100 pg/ml) of the glucan could be detected in all the samples examined. In contrast, high values (182–4,524 pg/ml) of the glucan concentrations were obtained in the plasmas from nine patients of deep mycosis.

Immunohistochemical Staining

Figure 3 shows immunohistochemical staining of T-GBP bound to (1→3)-β-D-glucan in the cell walls of *C. albicans* yeasts. Fluorescence due to FITC-protein A was located in the outer layer of the cells.

DISCUSSION

Hemocytes or amebocytes of the horseshoe crabs have been reported to contain various proteins that play crucial roles to defend themselves from bacterial and fungal infections (13). A number of such antimicrobial proteins were isolated from the amebocytes and plasma of the Japanese horseshoe crab, *T. tridentatus*, and their structures and functions have been elucidated (13). Two protease zymogens, factors C and G, in the amebocytes are converted to their active forms by specific binding to LPSs (gram-negative bacterial endotoxins) and (1→3)-β-D-glucans, (a major cell wall constituent of fungi), respectively, thereby initiating the coagulation pathway that protects the invasion of the pathogens. In addition, it has been found that the amebocyte contain a novel (1→3)-β-D-glucan-binding protein, T-GBP, which may act as an important member of host-defense system against fungous attack.

In this work, using T-GBP and anti-(1→3)-β-D-glucan antibody, we have developed a sandwich ELISA (GBP-ELISA), which is able to detect as little as 0.1 nanogram of the (1→3)-β-D-glucan with a high precision (CV < 10%) and covers a wide measurable range of concentrations (0.1–1,000 ng/ml). This method can be applied to quantification of (1→3)-β-D-glucan, regardless of linear and branched type, in accordance with the binding specificity of T-GBP, which does not react totally with glycans differing in the type of linkages, mannan, LPSs, and peptidoglycan. Another distinctive feature of the method is its applicability to the assay for such (1→3)-β-D-glucans with low molecular weights ($M_n < 5,800$) as laminarins, which eludes the method using factor G (14).

Deproteinization of plasma samples by treatment with per-

TABLE 2. Plasma (1→3)-β-D-Glucan Concentrations in Clinical Samples

Case	No	Clinical notes ^a	Age (yr)	Sex	(1→3)-β-D-Glucan (pg/ml)
Fungal infection	1	<i>Candida albicans</i>	74	M	560
	2	<i>Candida albicans</i>	82	F	4524
	3	<i>Candida guilliermondii</i>	66	M	3208
	4	<i>Candida tropicalis</i>	39	M	1003
	5	<i>Candida glabrata</i>	27	F	345
	6	<i>Aspergillus fumigatus</i>	43	F	182
	7	<i>Aspergillus flavus</i>	67	M	389
	8	<i>Cryptococcus neoformans</i>	72	M	450
	9	<i>Cryptococcus neoformans</i>	28	M	255
Other bacterial infection	10	<i>Pseudomonas aeruginosa</i>	70	F	ND
	11	<i>Pseudomonas cepacia</i>	59	F	ND
	12	<i>Klebsiella pneumoniae</i>	47	F	ND
	13	<i>E. coli</i> spp.	55	M	ND
	14	<i>Staphylococcus epidermidis</i>	49	F	ND
	15	<i>Streptococcus</i> spp.	40	M	ND
Normal	16–25				ND

^aOf the 9 fungal infections, 5 candidiasis and 1 cryptococcosis (8) yielded blood cultures positive. Two aspergillus pneumonia and 1 cryptococcosis were verified at necropsy. Of the 6 bacterial infections (bacteremia), 4 gram-negative bacterial sepsis and 2 gram-positive bacterial pneumonia. ND = not detectable.

chloric acid and assay of the supernatants by GBP-ELISA resulted in quantitative recoveries of varying concentrations of pachyman added to plasmas from human and various animals, as obtained by chromogenic limulus test (Gluspey) (5). It is apparent that GBP-ELISA is much more rapid and sensitive procedure than blood culture for detecting infection of mice with *C. tropicalis*. Correlation between the level of plasma (1→3)-β-D-glucan and the numbers of viable yeast cells in the kidney, a main target organ of the pathogen, indi-

cates that this method may provide a reliable estimate for the severity of fungal infection. Furthermore, data for rapid disappearance of plasma (1→3)-β-D-glucan in *C. tropicalis*-infected mice after infusion of amphotericin B implicates that GBP-ELISA is applicable to assess the effect of antifungals in vivo efficiently. Similar assay of clinical samples by GBP-ELISA revealed that higher values of plasma (1→3)-β-D-glucan matched definitely the diagnosis of fungal infection, whereas negative reaction was obtained with plasmas from normal subjects and patients of bacteremia. From these results, it is promising that GBP-ELISA has many potential uses in clinical as well as experimental medicine, in view of high binding specificity of T-GBP towards various (1→3)-β-D-glucans, although it is less sensitive as compared to chromogenic assay using factor G and the coagulation enzyme system.

Immunohistochemical microscopy using T-GBP and rabbit anti-T-GBP antibody provides a highly sensitive method to localize (1→3)-β-D-glucans in the cell walls of *C. albicans* yeast cells, which can be detected by fluorescence due to FITC-protein A bound to the antibody against T-GBP. Thus this method can be extended to study the localization and distribution of the glucans containing consecutive alignments of (1→3)-β-D-glucosyl residues in infected cells and tissues of fungemia as well as of eumycetes and possibly plants, just as factor C has been used to detect the in vivo distribution of endotoxin (15).

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Fig. 3. Immunohistochemical staining of (1→3)-β-D-glucan layer in the cell wall of *Candida albicans* cells. (magnification, ×75).

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