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Plasma (1→3)-β-D-Glucan and Fungal Antigenemia in Patients with Candidemia, Aspergillosis, and Cryptococcosis

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(1→3)-β-D-Glucan is one of the major structural components of fungi, and it seems that it can be detected by the fractionated (1→3)-β-D-glucan-sensitive component from a *Limulus* lysate, factor G. We evaluated the concentration of (1→3)-β-D-glucan by using factor G and other fungal antigens in 24 patients with clinical evidence of mycosis and 36 healthy subjects. The mean concentration of (1→3)-β-D-glucan in the plasma of the healthy subjects was found to be 2.7 ± 1.9 pg/ml (range, <6.9 pg/ml), and it was found to be substantially higher in all 11 patients with candidemia (mean, 2,207.4 pg/ml; range, 325.4 to 8,449.0 pg/ml). Eight of those 11 patients with candidemia (73%) were positive for the Cand-Tec heat-labile candida antigen and only 3 patients (27%) were positive for mannan antigen. Three patients with invasive pulmonary aspergillosis were positive for galactomannan and had, in addition, high concentrations of (1→3)-β-D-glucan (mean, 323.3 pg/ml; range, 27.0 to 894.0 pg/ml). All 10 patients with cryptococcosis (including 2 patients with probable cryptococcosis) were positive for cryptococcal antigen by the Eiken latex test; however, (1→3)-β-D-glucan levels were not elevated in these patients (mean, 7.0 pg/ml; range, <16.5 pg/ml). Our results indicated that (1→3)-β-D-glucan levels are elevated in patients with candidiasis and aspergillosis but not in those with cryptococcosis.

The conventional *Limulus* test used for the detection of bacterial endotoxin is not just specific for endotoxin, because it has been reported to be sensitive to low concentrations of (1→3)-β-D-glucan, a major structural component of fungal cell walls (9). Fractionation of the *Limulus* amoebocyte lysate has shown it to be composed of two pathways (16). One contains factors B and C, which are sensitive to endotoxin, while a second pathway contains factor G, which is sensitive to (1→3)-β-D-glucan. This is confirmed by the fact that factor G-free *Limulus* lysates are endotoxin specific and do not react with (1→3)-β-D-glucan (19). Thus, the difference in titers between the conventional *Limulus* test and the endotoxin-specific test is a useful, although indirect, indicator for detecting (1→3)-β-D-glucan. We have reported previously that the conventional *Limulus* test reacts with both curdlan [linear (1→3)-β-D-glucan] and culture supernatants of *Candida albicans* (15). We also demonstrated that the difference in titers between the two *Limulus* tests, termed the fungal index (6), increases in a rabbit model of systemic candidiasis (15) and in patients with candidiasis (10).

The G test is a direct method for the detection of (1→3)-β-D-glucan that uses (1→3)-β-D-glucan-sensitive factor G (19). The G test has been carefully evaluated in our laboratory with the plasma of rabbits with experimentally induced systemic candidiasis (13). In that study, all rabbits tested positive by the G test, with higher titers than those obtained by assays for the detection of mannan antigenemia being demonstrated. Furthermore, the G test appeared to be more sensitive than the fungal index, and the correlation between both methods was exponential (13). The apparent increased sensitivity of the G test in detecting (1→3)-β-D-glucan suggested that the test might be useful for the early and rapid diagnosis of invasive candidiasis in humans. In the study described here, we compared the concentrations of (1→3)-β-D-glucan and other fungal antigens in patients with candidemia, aspergillosis, and cryptococcosis.

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MATERIALS AND METHODS

Subjects. Plasma samples were collected from 24 human immunodeficiency virus-negative patients with mycosis admitted to the Nagasaki University Hospital. Two weeks prior to plasma collection, all human immunoglobulin products or immunoactive β-glucans, such as lentinan, krestin, and schizophyllan, were discontinued. Plasma samples were also collected from 36 healthy volunteers (18 males and 18 females; age range, 20 to 50 years) who had not received any human immunoglobulin products or immunoactive β-glucans during their lifetimes.

Candidemia was diagnosed on the basis of at least two blood cultures positive for *Candida* spp., together with a temperature of more than 38.0°C which was refractory to antibiotics for more than 3 days. Routine blood cultures (lysis-centrifugation and Roche Septi-Chek biphasic media) were performed as described previously (10). *C. albicans* was detected in one patient, *Candida parapsilosis* was detected in four patients, *Candida guilliermondii* was detected in two patients, *Candida krusei* was detected in one patient, and *Candida* (*Torulopsis*) *glabrata* was detected in three patients (see Table 1). When more than one sample was collected for the G test, the first sample was used for evaluation.

Seven patients with cultures of sputum, bronchoalveolar lavage fluid, or specimens obtained by transbronchial lung biopsy positive for *Cryptococcus neoformans* were diagnosed as having pulmonary cryptococcosis (see Table 2). Cryptococcal meningitis or disseminated cryptococcosis was not observed in these seven patients. Two patients (patients 8 and 9; see Table 2) with positive Eiken latex agglutination test results for cryptococcal antigen (11, 21) were suspected of having pulmonary cryptococcosis, but biopsy and culture results for these patients were negative. They improved clinically after treatment with intravenous fluconazole. Another patient (patient 10; see Table 2) had adult T-cell leukemia/lymphoma and was confirmed to have cryptococcal meningitis by a positive culture of the cerebrospinal fluid.

Three patients with hematological malignancies were diagnosed as having invasive aspergillosis on autopsy (see Table 3). Histological examination demonstrated characteristic hyphae in several organs including the lungs. Other types of invasive mycotic infections were not detected on autopsy. Cultures of blood from those patients were also negative for other organisms, including *Candida* spp.

G test. As mentioned previously, the G test contains a horseshoe crab coagulation cascade that is extremely sensitive and specific to (1→3)-β-D-glucan and a chromogenic substrate, Boc–Leu–Gly–Arg–*p*-nitroanilide (Seikagaku Kogyo Corp., Tokyo, Japan) (13). Factor G is activated by (1→3)-β-D-glucan, leading to activation of the proclotting enzyme. The chromogenic substrate is cleaved by

TABLE 1. (1→3)-β-D-Glucan concentrations in patients with candidemia

Patient no.	Underlying disease	IVH[a]	Blood culture	(1→3)-β-D-Glucan concn (pg/ml)	Cand-Tec[b]	Pastorex[c]
1	Miliary tuberculosis	+	*C. albicans*	521.0	8×	
2	Cerebral hemorrhage	+	*C. parapsilosis*	325.4	4×	
3	Hepatoma	−	*C. parapsilosis*	402.1	4×	
4	Cerebral infarction	+	*C. parapsilosis*	436.5		1×
5	Respiratory failure	+	*C. parapsilosis*	692.0	4×	
6	Heart failure	+	*C. guilliermondii*	565.0	8×	
7	Lung cancer	−	*C. guilliermondii*	1,485.1	4×	
8	Cerebral infarction	+	*C. glabrata*	758.5	8×	
9	Stomach cancer	−	*C. glabrata*	3,166.0	4×	16×
10	Cerebral infarction	+	*C. glabrata*	7,480.4	2×	2×
11	ATL, BMT[d]	−	*C. krusei*	8,449.0	2×	

[a] IVH, intravenous hyperalimentation.
[b] Values are reciprocal titer.
[c] Pastorex *Candida* assay; values are reciprocal titer.
[d] ATL, adult T-cell leukemia/lymphoma; BMT, bone marrow transplantation.

activated clotting enzyme; this is followed by the release of *p*-nitroanilide (19). We used endotoxin- and (1→3)-β-D-glucan-free glassware and plasticware purchased from Seikagaku Kogyo Corp. Plasma samples (100 μl) were treated with 0.32 mol of perchloric acid per liter (200 μl) at 37°C for 20 min. The mixture was precipitated by centrifugation at 1,500 × *g* for 10 min, and the supernatant (150 μl) was neutralized with 0.18 N NaOH (150 μl) before use in the G test. A 100-μl sample was added to 100 μl of G test reagents dissolved in 0.2 mol of Tris-HCl buffer (pH 8.0) per liter, and the mixture was then incubated at 37°C for 30 min. The released *p*-nitroaniline was converted into the diazo dye to enhance the sensitivity of the test. This was accomplished by adding 0.5 ml of each of the following reagents: 0.04% (wt/vol) sodium nitrite in 0.48 mol of HCl per liter, 0.3% (wt/vol) ammonium sulfate, and 0.07% (wt/vol) *N*-1-naphthylethylenediamine dihydrochloride (19). The A_{545} was determined. Samples were diluted when the A_{545} was more than 0.7 in order to obtain a good correlation between the (1→3)-β-D-glucan concentration and the absorbance (13). (1→3)-β-D-Glucan extract from *Poria cocos* was used as a standard. The G test was able to detect as little as 1.0 pg of (1→3)-β-D-glucan per ml, and the coefficient of variation was less than 5% (13). Duplicate assays were performed for each sample, and the mean values of duplicate tests are reported. In the statistical analysis, the concentration below the limit of measurement was considered to be 1.0 pg/ml.

Detection of fungal antigens. Mannan antigen from *Candida* spp. and heat-labile *Candida* antigen were determined by the Pastorex *Candida* (Diagnostics Pasteur, Marnes-la-Coquette, France) and the Cand-Tec (Ramco Laboratories, Inc., Houston, Tex.) assays, respectively (4). The Eiken latex test (Eiken, Tokyo, Japan) was used to detect cryptococcal antigen (11, 21), and the Pastorex *Aspergillus* assay (Diagnostics Pasteur) was used to detect galactomannan antigen (22). Twofold serial dilutions of plasma were tested to determine the antigen titer, which is defined as the reciprocal of the highest dilution in plasma with positive agglutination. All assays were performed according to the manufacturer's instructions. The concentration of galactomannan was also measured by an enzyme-linked immunosorbent assay (ELISA) method by using the avidin-biotin method (AB-ELISA). Briefly, New Zealand White rabbits were immunized with galactomannan antigens purified from *Aspergillus fumigatus*, and the resulting antigalactomannan immunoglobulin G (IgG) was fractionated with ammonium sulfate and DEAE-cellulose from the rabbit immune serum. Conjugation of biotin and streptavidin-alkaline phosphatase and the actual AB-ELISA test were performed as described previously (17). In our hands the detection level of the galactomannan concentration by the AB-ELISA was 2.5 ng/ml.

RESULTS

(1→3)-β-D-Glucan and mannan antigen concentrations in and Cand-Tec test results for patients with candidemia. The different concentrations of (1→3)-β-D-glucan in 11 patients with candidemia are given in Table 1. A high concentration of (1→3)-β-D-glucan was detected in each patient with candidemia, with the mean level for the group being 2,207.4 ± 2,967.8 pg/ml (range, 325.4 to 8,449.0 pg/ml). In contrast, the concentration of (1→3)-β-D-glucan in healthy volunteers was 2.7 ± 1.9 pg/ml (range, <6.9 pg/ml). The concentration of (1→3)-β-D-glucan in patients with candidemia was significantly elevated compared with that in healthy volunteers ($P < 0.01$ by Student's *t* test). Eight of these patients (73%) tested positive by the Cand-Tec test, and three patients (27%) tested positive by the Pastorex *Candida* assay, while the concentration of (1→3)-β-D-glucan was elevated in all patients when the cutoff value was set at 10.0 pg/ml.

(1→3)-β-D-Glucan concentration and capsular antigen in patients with cryptococcosis. The Eiken latex test for crypto-

TABLE 2. (1→3)-β-D-Glucan concentrations in patients with cryptococcosis

Patient no.	Underlying disease	Culture	(1→3)-β-D-Glucan concn (pg/ml)	Eiken latex assay
1	Lymphadenopathy (unknown etiology)	Sputum	11.8	8×
2	Old pulmonary tuberculosis	Sputum	4.7	1×
3	Diabetes mellitus	Sputum	9.6	1×
4	Diabetes mellitus	BALF[a]	<1.0[b]	1×
5	Renal failure, hemodialysis	BALF	16.5	16×
6	Rheumatoid arthritis	TBLB[c]	1.7	8×
7	Diabetes mellitus	Sputum	6.2	1×
8	None		1.6	1×
9	None		6.3	32×
10	ATL[d]	CSF[e]	10.6	1,024×

[a] BALF, bronchoalveolar lavage fluid.
[b] Below detection limit.
[c] TBLB, transbronchial lung biopsy.
[d] ATL, adult T-cell leukemia/lymphoma.
[e] CSF, cerebrospinal fluid.

TABLE 3. (1→3)-β-D-Glucan concentrations in patients with invasive aspergillosis

Patient no.	Underlying disease	Day	(1→3)-β-D-Glucan concn (pg/ml)	Pastorex[a]	ELISA (ng/ml)
1	Acute myelocytic leukemia	0	894.0	1×	ND[b]
		2	516.0	8×	19.0
		4	939.0	32×	17.7
2	Hypoplastic leukemia	0	27.0	1×	7.4
		9	246.0	2×	15.0
3	Aplastic anemia	0	49.4	ND	<2.5[c]
		15	113.4	2×	11.2
		21	521.0	2×	12.2
		27	150.0	1×	ND

[a] Pastorex *Aspergillus* assay; values are reciprocal titer.
[b] ND, not done.
[c] Below detection limit (2.5 ng/ml).

coccal antigen was positive for all of 10 patients with cryptococcosis (including 2 patients with probable cryptococcosis). However, all patients with cryptococcosis showed very little or no rise in (1→3)-β-D-glucan concentrations (Table 2). The mean (1→3)-β-D-glucan concentration in 10 patients was 7.0 ± 5.1 pg/ml (range, <16.5 pg/ml).

(1→3)-β-D-glucan and galactomannan concentrations in patients with invasive aspergillosis. All patients with leukopenia and evidence of invasive aspergillosis had elevated concentrations of (1→3)-β-D-glucan when they were initially examined (mean, 323.3 pg/ml; range, 27.0 to 894.0 pg/ml) (Table 3). The concentrations of galactomannan and (1→3)-β-D-glucan increased rapidly in all patients.

DISCUSSION

Mannan is the most widely studied antigen in patients with candidiasis. Various serological methods have been used to detect the level of the circulating mannan antigen. In the present study, we used the Pastorex *Candida* assay, a monoclonal antibody-based latex agglutination assay for mannan antigen that is available commercially and that is the most widely used test for mannan antigen detection (4). The Pastorex *Candida* assay reacts with mannan from *C. albicans*, *Candida tropicalis*, *C. glabrata*, *C. guilliermondii*, *C. parapsilosis*, and *Candida pseudotropicalis* (4), but it does not cross-react with mannan from *C. krusei* and is therefore not useful in the serological diagnosis of *C. krusei* infection. This is in contrast to a report by Nakamura et al. (17) that detection of mannan antigens of *C. glabrata* and *C. krusei* by AB-ELISA is difficult. All 11 patients in the present study, including a single patient infected with *C. krusei*, had high concentrations of (1→3)-β-D-glucan. Although the G test failed to detect the specific species of *Candida*, it appeared to be the most sensitive test for the serodiagnosis of candidiasis (13).

However, in the present study, all human immunodeficiency virus-negative patients with cryptococcosis had low concentrations of (1→3)-β-D-glucan. Therefore, the G test is not useful for the serodiagnosis of cryptococcosis. Results from our laboratory indicate that *C. albicans* and *A. fumigatus* release soluble (1→3)-β-D-glucan into culture fluids in parallel with their growth, but *C. neoformans* and *Mucor* spp. are unable to release soluble (1→3)-β-D-glucan or release very small amounts of soluble (1→3)-β-D-glucan into culture fluids (14). These data are in agreement with the results described in this report.

Invasive pulmonary aspergillosis in granulocytopenic patients presents as acute pneumonia. The infection progresses by hematogenous spread as well as extension to the surrounding lung structures. Since *A. fumigatus* releases (1→3)-β-D-glucan into the culture fluids in parallel with fungal growth (14) and the invasive form of the same fungus directly invades the pulmonary parenchyma and vasculature, it follows that galactomannan as well as (1→3)-β-D-glucan should be released into the bloodstream as well. Indeed, high concentrations of (1→3)-β-D-glucan have been detected in rats with experimentally induced aspergillosis (12), and were present in three of three patients diagnosed as having invasive aspergillosis.

False-positive reactions in the conventional *Limulus* test have been detected in patients undergoing hemodialysis (18) and those treated with human immunoglobulin products (7). The possible explanations for these observation is that the cellulose-based hemodialysis membrane is contaminated with (1→3)-β-D-glucan, as is the filter membrane used during the process of manufacturing immunoglobulin products (7). The mean concentration of (1→3)-β-D-glucan in nine patients undergoing hemodialysis with cellulose-based dialyzing membranes was 1,976.3 ± 1,183.2 pg/ml (unpublished data). The results of the G test must therefore be interpreted with caution for patients undergoing hemodialysis. However, the use of triacetate-based hemodialysis membranes avoids contamination with (1→3)-β-D-glucan. The mean concentration of (1→3)-β-D-glucan in seven patients who underwent hemodialysis with triacetate membranes and who had no evidence of invasive mycosis was 19.5 ± 13.9 pg/ml (unpublished data).

Janusz et al. (8) reported that human monocytes possess β-glucan receptors that initiate phagocytosis of glucan particles, on the basis of their observation that soluble β-glucan inhibited monocyte phagocytosis. On the basis of that observation and the results of our study, a possible mechanism for establishing a fungal infection might be that fungi release soluble β-glucan which blocks the β-glucan receptors of phagocytes, permitting the fungus to escape host defense mechanisms. In addition, (1→3)-β-D-glucan activates the alternative complement pathway (1, 3), stimulates leukotriene synthesis by monocytes (2), and induces the release of tumor necrosis factor alpha from monocytes in patients with *Pneumocystis carinii* infection (5). This wide range of effects on the human immune system (20) suggests that further studies are required to determine the potential roles of (1→3)-β-D-glucan during interaction of host defense mechanisms with fungal organisms.

In conclusion, the G test is a good method not only for the detection of deep mycosis in serum but also for evaluating the net effect of (1→3)-β-D-glucan on host inflammatory responses during fungal infections.

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