

## Comparison Between Wako-WB003 and Fungitec G Tests for Detection of (1→3)-β-D-Glucan in Systemic Mycosis

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The limulus factor G reacts with (1→3)-β-D-glucan, a major structural component of fungal cell walls. The Fungitec G test is a colorimetric assay that measures the concentration of (1→3)-β-D-glucan and is used as a serodiagnostic test for deep mycosis. Wako-WB003 is another assay for (1→3)-β-D-glucan that determines the change in turbidity of the gelatin reaction of limulus factor G with (1→3)-β-D-glucan. In five rabbits inoculated intravenously with  $1 \times 10^7$  CFU of *Candida albicans*, the concentration of (1→3)-β-D-glucan measured by the fungitec G test increased gradually reaching a peak of  $660.9 \pm 427.9$  pg/ml (mean  $\pm$  SD) 4 days after inoculation, but to  $42.225 \pm 41.275$  ng/ml on day 6 in the Wako-WB003 test. In one rabbit challenged intravenously with  $5 \times 10^6$  CFU of *C. albicans*, (1→3)-β-D-glucan increased to 101.5 pg/ml on day 4 on the fungitec G test, whereas the level remained below the detection limit of the Wako-WB003 test throughout the course of the disease. We also detected high concentrations of

(1→3)-β-D-glucan in 11 patients with candidemia, 4 with suspected candidemia, 1 with invasive pulmonary aspergillosis, and 12 patients with aspergilloma. The concentration of (1→3)-β-D-glucan measured by the Fungitec G test was  $> 150$ ,  $> 1006.8$ , 312.1, and  $55.6 \pm 37.4$  pg/ml (range, 20.1–138.0 pg/ml), and by the Wako-WB003 test  $> 153.000$ ,  $> 17.70$ , 153.000 and  $2.645 \pm 7.248$  ng/ml (range,  $< 25.20$  ng/ml) in these patients, respectively. In contrast, the concentration of (1→3)-β-D-glucan in 9 patients with pulmonary cryptococcosis and 6 with superficial candida colonization ranged from  $< 13.2$  and  $< 15.3$  pg/ml in the Fungitec G test and  $< 0.53$  and  $< 0.12$  ng/ml in Wako-WB003 test. There was a weak relationship between the concentration of (1→3)-β-D-glucan measured by the Fungitec G test and Wako-WB003 test ( $r = 0.521$ ). Our results indicate that the sensitivity of the Wako-WB003 test is lower than that of the Fungitec G test. J. Clin. Lab. Anal. 11: 73–77. © 1997 Wiley-Liss, Inc.

**Key words:** Fungitec G test; Wako-WB003 test; (1→3)-β-D-glucan; candidiasis; aspergillosis; diagnostic test

### INTRODUCTION

Systemic candidiasis is a significant complication in neutropenic patients with hematological malignancies, cancer patients undergoing chemotherapy, and in individuals with immunosuppressive states such as AIDS. Individuals receiving steroids on a long-term basis or those with intravenous catheters are also at increased risk of developing this infection.

Although the lysis centrifugation technique is a rapid and sensitive method for the diagnosis of candidemia, blood cultures may be negative in a considerable number of patients with invasive candidiasis. Since early recognition and management of candidiasis are two important factors that reduce candidiasis-related mortality, other more sensitive and specific methods need to be established to provide a simple and fast detection of the fungus. One such approach involves the detection of fungal antigens or metabolic products in the se-

rum (1). Mannan is the most widely studied antigen in patients with candidiasis. Various serological methods have been used to detect circulating mannan antigen (2, 3). However, recent studies revealed that plasma from patients with candidiasis contains (1→3)-β-D-glucan, another major structural component of fungal cell wall (3–5). Accordingly, the fungitec G test, referred to previously as the G test, was developed to measure plasma concentrations of (1→3)-β-D-glucan. We have already reported that the test was positive in animals with systemic candidiasis (6). Furthermore, the test was faster in establishing the diagnosis and yielded higher concentrations of (1→3)-β-D-glucan compared with mannan

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Received 26 January 1996; Accepted 5 September 1996

antigenemia. We have also reported that several fungi, including *Candida albicans* and *Aspergillus fumigatus*, released soluble (1→3)-β-D-glucan into culture fluids, whereas *Cryptococcus neoformans* and *Mucor spp.* released only small amounts of soluble (1→3)-β-D-glucan into culture fluids (16). Furthermore, our early clinical results in patients with blood cultures positive for *Candida spp.* showed high sensitivity rates, with all 11 patients tested positive (13). Our results demonstrated also that the mean plasma concentration of (1→3)-β-D-glucan in patients with candidemia (mean, 2207.4 pg/ml; range, 325.4 to 8449.0 pg/ml) was substantially higher than that of healthy subjects ( $2.7 \pm 1.9$  pg/ml, range, < 6.9 pg/ml). In addition, high concentrations of (1→3)-β-D-glucan were detected in experimentally induced aspergillosis (17) and in patients with invasive pulmonary aspergillosis, but not in patients with cryptococcal infection (13).

The Wako-WB003 is another assay for measuring (1→3)-β-D-glucan. In this study, the concentration of (1→3)-β-D-glucan was measured simultaneously using the Wako-WB003 and the Fungitec G tests. We also compared the sensitivity of the two assays in detecting candidiasis in rabbits with experimental systemic candidiasis and patients with deep mycosis.

## MATERIALS AND METHODS

### Patients

Plasma samples were collected from 37 patients with deep mycosis admitted to the Nagasaki University Hospital. Blood cultures were positive for *Candida* species in 11 patients. One patient with acute leukemia was found to have invasive pulmonary aspergillosis at autopsy. Twelve patients were radiologically diagnosed with aspergilloma, with positive cultures of bronchoalveolar lavage fluid and/or sputum for *A. fumigatus*. Nine patients were diagnosed with cryptococcosis confirmed on lung biopsies or cultures of BALF to be positive for *C. neoformans*. Four febrile patients were suspected to have candidemia, who underwent intravenous hyperalimentation and were refractory to antibiotics. These four patients showed negative cultures of *Candida* species, but improved clinically after treatment with intravenous fluconazole.

Plasma samples were also obtained from six patients with superficial candida colonization. None of these six patients had a positive blood culture for *Candida* species or other organisms. *Candida* species were recovered from oral thrush, skin lesions or from urine samples.

Endotoxin- and (1→3)-β-D-glucan-free glassware and plasticware, purchased from Seikagaku Kogyo Corp. (Tokyo, Japan), were used in this study. Plasma samples were collected and stored at -80°C until use.

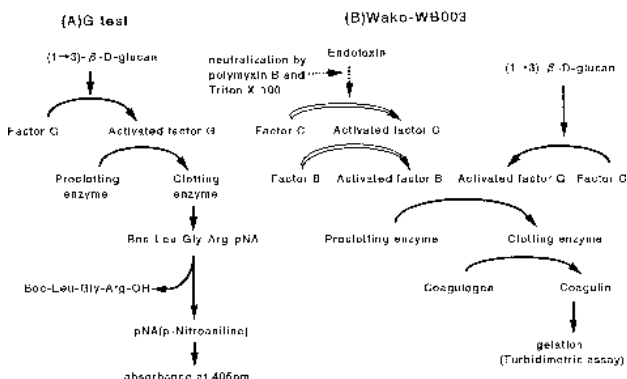
### Rabbit Model of Systemic Candidiasis

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation at our insti-

tion. A rabbit model of systemic candidiasis was prepared by intravenous inoculation of *C. albicans* strain IFM 40009. The strain was cultured at 30°C for 24 hours in Sabouraud Dextrose Agar. After washing for three times with sterile saline, 1.0 ml of cell suspension was injected into the tail vein of each rabbit. Each of five Japanese White rabbits was inoculated with  $1 \times 10^7$  CFU of *C. albicans*. A different dose of *C. albicans* ( $5 \times 10^6$  CFU) was also inoculated in another single rabbit. Heparinized blood was collected before inoculation of the fungus and on days 2, 4, 6, 9, 13, and 21 after inoculation.

### Wako-WB003 Test

The *Limulus* amoebocyte lysate (LAL) is composed of two coagulation pathways as demonstrated by fractionation analysis (7–9). The first pathway contains factors B and C that are sensitive to endotoxin, whereas the second pathway contains factor G, which is sensitive to (1→3)-β-D-glucan. The Wako-WB003 test (Wako Pure Chemical Industries, Osaka, Japan) is based on the endotoxin-neutralizing effect of the pretreatment solution (polymyxin B and Triton X-100) (Fig. 1B). In this test, 100 μl of plasma was added to (1→3)-β-D-glucan-free tubes containing 900 μl of pretreatment solution (0.2% triton X-100, 0.002% polymyxin B). Pretreatment was performed at 80°C for 10 min to neutralize any contaminating endotoxin. After keeping on ice for 5–10 min, 100 μl sample of the supernatant of pretreated samples was mixed with 100 μl of LAL reagent. In this process, (1→3)-β-D-glucan triggers the gelation reaction of factor G, which in turn increases the turbidity of the reaction mixture. Increased turbidity was measured with the kinetic turbidimetric method using Toxinometer MT 251 (Wako Pure Chemical Industries). The gelation time (T<sub>g</sub>) was defined as the reaction time required for the transmittance to diminish to threshold values (98% of the initial transmittance). Thus the higher the (1→3)-β-D-glucan concentration in the sample, the shorter the gelation time, and  $\log[(1 \rightarrow 3)\text{-}\beta\text{-D-glucan}]$  is inversely proportionate



**Fig. 1.** Schematic diagram of the steps involved in (A) the Fungitec G test, and (B) Wako-WB003 test. The endotoxin-specific pathway in Wako-WB003 test can be inhibited by the pretreatment solution (polymyxin B and Triton X-100).

with log(Tg). We also used curdlan, a linear (1→3)-β-D-glucan produced by *Alcaligenes faecalis* var. *myxogenes* IFO 13140, as a standard.

**Fungitec G Test**

The Fungitec G test (Seikagaku Kogyo) is specific to (1→3)-β-D-glucan, since factors B and C are eliminated in the test (10, 11). Factor G is activated by (1→3)-β-D-glucan, leading to activation of the pro-clotting enzyme. In this test, the chromogenic substrate, Boc-Leu-Gly-Arg-p-nitroanilide, is cleaved by the activated clotting enzyme (9), followed by the release of p-nitroanilide. The released p-nitroanilide is determined at absorbance at 405 nm (Fig. 1A).

A 5.0 μl of plasma sample was pretreated with 20 μl of the test solution containing 0.15 M KOH, 0.3 M KCl, and 0.1% polybrene, and the mixture was incubated for 10 min at 37°C. The pretreated sample was added to 100 μl of factor G dissolved in HEPES buffer (0.1 M, pH 7.6), and then incubated at 37°C for 30 min. The optical density at 405 nm was measured using the kinetic mode of a computerized Well-reader SK601 (Seikagaku Kogyo). Pachyman, (1→3)-β-D-glucan from *Poria cocos*, was used as a standard. The coefficient of variation in the fungitec G test was < 5% (6). Duplicate assays were performed in each sample, and the average of the two tests was reported.

**Statistical Analysis**

Concentrations below the detection limit of the test (0.075 ng/ml for the Wako-WB003 test, and 1.0 pg/ml for the Fungitec G test) were approximated to 0.01 ng/ml and 0.1 pg/ml, respectively. Data were expressed as mean ± SD. The relationship between the concentration of (1→3)-β-D-glucan measured by the two tests was examined using the linear regression analysis. The χ<sup>2</sup> test was also used to compare the results of the two tests.

**RESULTS**

**(1→3)-β-D-Glucan Concentration in Rabbits**

Intravenous inoculation of 1 × 10<sup>7</sup> CFU of *C. albican* in five rabbits resulted in the death of two animals after 4 days, one after 6 days, another after 9 days, and only one animal was still alive after 21 days. The concentrations of (1→3)-β-D-glucan measured on specific days in these animals, using the Wako-WB003 and Fungitec G tests, are shown in Table 1. The concentrations of (1→3)-β-D-glucan increased gradually and reached a peak of 42.225 ± 41.275 ng/ml on day 6 as measured by Wako-WB003, and 660.9 ± 427.9 pg/ml on day 4 on the Fungitec G test.

The single rabbit challenged with an intravenous dose of 5 × 10<sup>6</sup> CFU of *C. albicans* survived for > 21 days. The concentration of (1→3)-β-D-glucan in this animal increased gradually from 7.0 pg/ml on the control day to 101.5 pg/ml 4

**TABLE 1. (1→3)-β-D-Glucan in Rabbits With Systemic Candidiasis**

| Day                            | Wako-WB003 (ng/ml) <sup>a</sup> | G test (pg/ml) |
|--------------------------------|---------------------------------|----------------|
| 1 × 10 <sup>7</sup> CFU/rabbit |                                 |                |
| 0                              | —                               | 7.0 ± 1.2      |
| 2                              | 10.505 ± 13.787                 | 116.7 ± 80.8   |
| 4                              | 18.802 ± 14.668                 | 660.9 ± 427.9  |
| 6                              | 42.225 ± 41.275                 | 573.4 ± 431.0  |
| 9                              | 0.772 ± 0.548                   | 102.9 ± 24.7   |
| 21                             | 1.320                           | 39.7           |
| 5 × 10 <sup>6</sup> CFU/rabbit |                                 |                |
| 0                              | —                               | 7.0            |
| 2                              | —                               | 49.0           |
| 4                              | —                               | 101.5          |
| 9                              | —                               | 25.8           |
| 14                             | —                               | 11.3           |

<sup>a</sup>— = < 0.075 ng/ml.

days after inoculation on the fungitec G test. In contrast, the concentration of (1→3)-β-D-glucan was not detected at all throughout the course of infection on the Wako-WB003 test, indicating that the sensitivity of the Wako-WB003 was lower than that of the Fungitec G test.

There was a weak relationship between the Fungitec G test and the Wako-WB003 test in experimental candidiasis (r = 0.630).

**(1→3)-β-D-Glucan Concentration in Patients**

(1→3)-β-D-glucan was measured in 37 patients with deep mycosis and six patients with superficial candida colonization (Table 2). The concentration was high in all 11 patients with candidemia (> 150 pg/ml by the Fungitec G test, and > 1.350 ng/ml by Wako-WB003). In a single patient with invasive pulmonary aspergillosis, the concentration of (1→3)-β-D-glucan was 312.1 pg/ml by the Fungitec G test, and 153.000 ng/ml by the Wako-WB003 test. However, there was a moderate or small rise in the concentration in 12 patients with aspergilloma in both tests (mean 2.645 ng/ml; range, < 25.20 ng/ml in Wako-WB003 test and mean, 55.6 pg/ml; range, 20.1 to 138.0 pg/ml in the fungitec G test). In contrast, the concentration of (1→3)-β-D-glucan in 9 patients with pulmonary cryptococcosis was very low or close to the minimum detec-

**TABLE 2. (1→3)-β-D-Glucan in Patients With Deep Mycosis**

| Diagnosis            | n  | Wako-WB003 (ng/ml) | G test (pg/ml) |
|----------------------|----|--------------------|----------------|
| Deep mycosis         |    |                    |                |
| Candidemia           | 11 | 22.398 ± 49.475    | 494.7 ± 448.4  |
| IPA <sup>a</sup>     | 1  | 153.00             | 312.1          |
| Aspergilloma         | 12 | 2.645 ± 7.248      | 55.6 ± 37.4    |
| Cryptococcosis       | 9  | 0.137 ± 0.170      | 5.5 ± 5.1      |
| Suspected candidemia | 4  | 126.575 ± 128.485  | 1416.8 ± 449.9 |
| SCC <sup>b</sup>     | 6  | 0.045 ± 0.054      | 7.9 ± 7.3      |

<sup>a</sup>Invasive pulmonary aspergillosis.

<sup>b</sup>Superficial candida colonization.

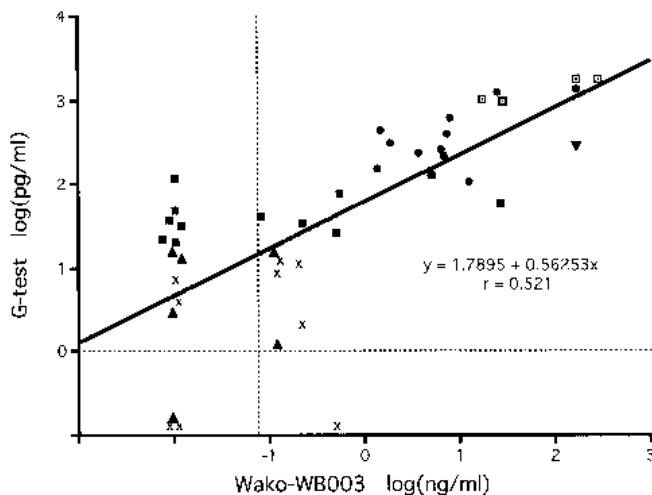
tion limit (mean, 0.137 ng/ml; range, < 0.53 ng/ml in Wako-WB003 test and mean, 5.5 pg/ml; range, < 13.2 pg/ml in the fungitec G test). Four patients with suspected candidemia had high concentrations of (1→3)-β-D-glucan detected by both assays. The concentration of (1→3)-β-D-glucan was > 17.70 ng/l in Wako-WB003 test and > 1006.8 pg/ml in the fungitec G test. Furthermore, the concentration of (1→3)-β-D-glucan in six patients with superficial candida colonization was < 0.12 ng/ml in Wako-WB003 test and < 15.3 pg/ml in the fungitec G test.

### Relationship Between Wako-WB003 and Fungitec G Tests

The relationship between the plasma concentration of (1→3)-β-D-glucan measured by the Wako-WB003 test and that by the Fungitec G test in patients with mycosis is shown in Figure 2. There was a weak relationship between the fungitec G test and the Wako-WB003 in these patients ( $r = 0.521$ ). (1→3)-β-D-glucan was detected in 39 of 43 (90.7%) patients by the Fungitec G test, and 29 (67.4%) patients by the Wako-WB003 test. The results were different in 12 of 43 patients, although these differences were not significant ( $\chi^2 = 3.631$  by  $\chi^2$  test).

### DISCUSSION

The limulus test is extremely sensitive to endotoxin and has been used to quantify nanogram quantities of the toxin. However, the conventional limulus test is not specific for endotoxin as it is also sensitive to low concentrations of



**Fig. 2.** Relationship between the concentration of (1→3)-β-D-glucan measured by the Wako-WB003 and Fungitec G tests in patients with deep mycosis. Concentrations below the detection limit of the test (0.075 ng/ml for the Wako-WB003 test, and 1.0 pg/ml for the fungitec G test) were approximated to 0.01 ng/ml and 0.1 pg/ml, respectively. Candidemia (●), Invasive pulmonary aspergillosis (▼), Aspergilloma (■), Cryptococcosis (X), Candidemia suspected (□), superficial candida colonization (▲).

(1→3)-β-D-glucan (7–10). The test was constructed with two pathways. The first contains factors B and C, which are sensitive to endotoxin, whereas the second contains factor G, which is sensitive to (1→3)-β-D-glucan (8). To improve the specificity of the conventional limulus test to endotoxin, Obayashi et al. (11) developed an endotoxin-specific chromogenic limulus test devoid of limulus factor G. The endotoxin-specific limulus test reacts only with the endotoxin and not with (1→3)-β-D-glucan. The difference between the conventional limulus test and endotoxin-specific limulus test, tentatively named the fungal index, is an indirect method for detecting (1→3)-β-D-glucan. Ikegami et al. (4) reported a rise in the fungal index in patients with candidiasis. Results from our laboratory confirmed that differences between titers measured by the two tests were higher in culture supernatants of *C. albicans* (12), plasma of rabbits with systemic candidiasis (12), and plasma of patients with candidemia (3).

Since measurement of the fungal index requires a laborious determination of the titer by the two tests, a simpler assay with a good sensitivity and specificity for (1→3)-β-D-glucan is desirable. The Fungitec G test is a direct method that detects (1→3)-β-D-glucan using a (1→3)-β-D-glucan-sensitive component, factor G, fractionated from LAL. We have recently examined the reliability of the Fungitec G test using plasma of rabbits with experimental systemic candidiasis (6) and patients with mycosis (13). The test was found to be true positive in all samples and the titers were higher than those measured by the mannan antigenemia. The sensitivity of the Fungitec G test surpassed that of the two limulus tests even when used together (6).

The Wako-WB003 is another method specific for (1→3)-β-D-glucan. This test is based on the endotoxin-neutralizing effect of polymyxin B and Triton X-100. The test differs from the Fungitec G test in that it contains limulus factors B and C. The Wako-WB003 is used currently in an automated format measuring the change in turbidity of the gelation reaction between the limulus factor G and (1→3)-β-D-glucan. Although the procedure is simpler than that of the fungitec G test, the present study demonstrated that the sensitivity of the Wako-WB003 test was lower than that of the Fungitec G test. Our results also demonstrated that the detection limit for curdlan in the Wako-WB003 test was 0.075 ng/ml. In contrast, the Fungitec G test is a very sensitive assay for the early detection of as little as 1.0 pg/ml of pachyman (6).

It also has been demonstrated that when exhibiting a single helical conformation of (1→3)-β-D-glucan, the Fungitec G test is more effective than taking a triple helical conformation for the activation of the limulus factor G (14). We used a mixture of KOH, KCl, and polybrene as the pretreatment solution in the Fungitec G test. Treatment with this alkaline solution could change the triple helical (1→3)-β-D-glucan to a single helical form (14). This is one possible reason for the high sensitivity of the Fungitec G test compared with the Wako-WB003 test, in which a dilution-heating procedure is

used as a pretreatment of the specimens. Furthermore, the correlation between the Fungitec G test and conventional limulus test was not linear but rather exponential (6), i.e., factor G reacts more effectively with (1→3)-β-D-glucan than intact LAL (factor G plus factors B and C). It is possible that this property is another reason for the enhanced sensitivity of the Fungitec G test compared with the Wako-WB003 test.

Zhang et al. (15) reported that the endotoxin-activated pathway in LAL can be differentially inhibited by either the combined use of dimethyl sulfoxide (DMSO) and polymyxin B, or a monoclonal antibody against limulus factor C. Based on the ability of polymyxin B to neutralize endotoxin, DMSO inhibition of factor C, and the neutralization of factor C by a specific monoclonal antibody against limulus factor C, these workers prepared two limulus peptide C ELISA specific for (1→3)-β-D-glucan. The detection limit for curdlan in the (1→3)-β-D-glucan-specific limulus ELISA was ~ 50 pg/ml. Surprisingly, the concentration of (1→3)-β-D-glucan in normal human plasma was 1.82 and 2.42 ng/ml using the two (1→3)-β-D-glucan-specific limulus ELISA, respectively (15). In addition, the concentration of (1→3)-β-D-glucan in normal human plasma was 0.17 ± 0.12 ng/ml (n = 24) using the Wako-WB003. In a previous study from our laboratory, the mean concentration of (1→3)-β-D-glucan in plasma of 36 normal volunteers, measured by the Fungitec G test, was 2.7 ± 1.9 pg/ml (range: < 6.9 pg/ml) (13). Although the exact reasons for the discrepancy between these two sets of data are difficult to explain, we suggest that the differences could be due to differences in the sensitivity and standard material used in each study.

The present results in patients with a variety of fungal infections are extension of those reported earlier and further indicate that the detection of (1→3)-β-D-glucan is a useful method for the diagnosis of systemic fungal infection, particularly in candidiasis and aspergillosis.

**ACKNOWLEDGMENTS**

The authors thank Assoc. Professor F.G. Issa from the Department of Medicine, University of Sydney, Sydney, Australia, for the critical reading and editing of the manuscript.

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