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(1→3) β-D-Glucan as a Quantitative Serological Marker for *Pneumocystis carinii* Pneumonia

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We detected (1→3) β-D-glucan (β-glucan), which is one of the major components of the cyst wall of *Pneumocystis carinii*, in sera obtained from patients with *P. carinii* pneumonia (PCP). We confirmed that β-glucan was detectable by a β-glucan detection kit (G test; Seikagaku Corporation) in bronchoalveolar lavage fluids (BALFs). The mean concentration of β-glucan in BALFs obtained from specific-pathogen-free nude mice infected with *P. carinii* ($n = 7$; mean, 2,631 [range, 1,031 to 9,095] pg/ml) was significantly higher ($P < 0.001$) than that in uninfected, specific-pathogen-free mice ($n = 7$; 6.5 [range, 4.0 to 8.3] pg/ml). The mean level of β-glucan in BALFs from PCP patients was significantly higher ($P < 0.05$) than that in BALFs from patients with other lung diseases (7,268 [range, 1,355 to 15,500] pg/ml [$n = 4$] versus 242.5 [17 to 615] pg/ml [$n = 4$]). In sera from six of seven patients with PCP, significant levels of β-glucan (494.1 [8.5 to 1,135] pg/ml) were detected, while it was undetectable in patients with other lung diseases and in a control group. In five patients at follow-up, the level of β-glucan decreased with clinical improvement. These results suggest that β-glucan is detectable in sera from patients with PCP and it is a practical serological marker for monitoring of the disease during treatment.

Several methods with satisfactory sensitivity and specificity have been established for the diagnosis of *Pneumocystis carinii* pneumonia (PCP), including conventional cytochemical staining, immunofluorescent staining with monoclonal antibodies (6), and the PCR (4, 15). However, none of the developed methods are quantitative. PCP is usually treated for 3 weeks in AIDS patients (9), despite the severity of the pneumonia, because there are no sensitive and quantitative markers for monitoring of disease activity during treatment. Thus, it is clinically important to develop a method for monitoring of the disease (13). A readily available and minimally invasive sample, such as blood, is desired.

(1→3) β-D-Glucan (β-glucan) is known to compose a portion of the cell wall of most fungi. Previous clinical investigations have shown that mycotic β-glucan can be detected in patients' sera. There was a good correlation between β-glucan levels and severity of disease (12) when detection of β-glucan in sera was used as a serological marker for the diagnosis of organic mycosis. Recently, *P. carinii* has been placed in a fungus family (1) and β-glucan has been detected in the cyst wall (10). These observations prompted us to examine the β-glucan levels in sera of patients with PCP. We report here that it was detectable in serum samples. Furthermore, to confirm whether or not β-glucan originated from *P. carinii*, we examined β-glucan levels of bronchoalveolar lavage fluids (BALFs) obtained from PCP patients and PCP-infected, specific-pathogen-free (SPF) nude mice.

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MATERIALS AND METHODS

BALFs from mice with PCP. Uninfected, SPF, female, nude mice (BALB/c *nu/nu*; 5 weeks old, weighing 14 to 18 g; Japan SLC, Shizuoka, Japan) were kept in an isolator (a plastic unit with laminar air flow and HEPA filtration) with SPF mice infected with *P. carinii* to establish infection as described previously (4). The mice were given disinfected water, food, and bedding to exclude infections from other pathogens, including fungi. Sixteen weeks later, seven mice infected with *P. carinii* by airborne transmission were sacrificed and exsanguinated. After making pneumothoraxes by incising the diaphragm, 22-gauge Teflon needles 1.5 in. (3.81 cm) long were inserted into the exposed trachea. The lungs were washed eight times by injecting 0.75 ml of β-glucan-free saline each time through the inserted needle. The collected BALFs, approximately 5 ml each, were centrifuged at 500 × *g* for 10 min at 4°C. The precipitates were resuspended with 200 μl of saline and stored at −80°C until measurement. Uninfected, SPF mice ($n = 7$) were maintained as controls, and BALFs were obtained from them in the same manner as described above.

Human BALFs. Eight BALFs were obtained consecutively (July 1993 to April 1994) from patients with AIDS and suspected lung diseases at the Hospital of the Institute of Medical Science, University of Tokyo, after obtaining informed consent. Four BALFs were confirmed histologically and clinically as coming from patients with PCP. For collection of BALFs, the target bronchus was washed twice with 30 ml of sterilized saline each time. The samples (20 to 40 ml) were separated into aliquots for microscopic examination and storage at −80°C.

Human sera. Human sera were obtained from seven consecutive patients with AIDS and PCP, four patients with AIDS and lung diseases other than PCP or fungal diseases, and five oral candidiasis patients (April 1993 to April 1994). PCP was diagnosed microscopically from BALFs or percutaneous lung aspiration samples. First samples were obtained within a week of antipneumocystis therapy. Time course samples were drawn from some patients. The patients who were diagnosed as having other lung diseases were evaluated by bronchoalveolar lavage examination. *P. carinii* and fungi were not detected in their BALFs. The oral candidiasis patients were all infected with human immunodeficiency virus but had no other opportunistic infections at the time blood was drawn. Control sera were collected separately from 14 human immunodeficiency virus-positive patients without opportunistic infections. All samples were treated with and stored in β-glucan-free materials.

β-Glucan determination. β-Glucan was measured with the G test (Seikagaku Corporation, Tokyo Japan), which was originally developed to detect fungal β-glucan for the diagnosis of deep mycosis (12). Manipulation was performed in accordance with the manufacturer's instructions. In brief, 5 μl of serum was incubated with 20 μl of an alkaline reagent (0.15 mol of potassium hydroxide per liter, 0.3 mol of potassium chloride per liter, 0.1% Polybrene, 0.02 mol of EDTA-4 NA per liter) in a 96-well β-glucan-free microplate (Toxipet plate; Seikagaku Corporation) at 37°C for 10 min. A 100-μl volume of β-glucan-specific

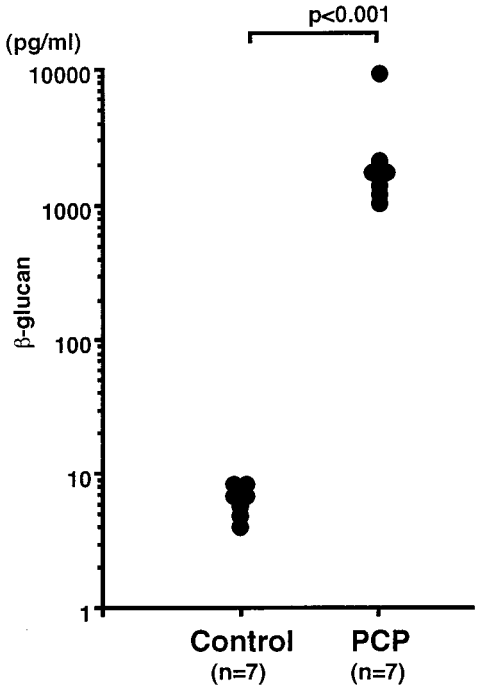

FIG. 1. β-Glucan levels in BALFs from mice. The *P* value was determined with the unpaired Wilcoxon test.

chromogenic *Limulus* reagent was then added to each well for kinetic measurement of the reaction at 37°C for 30 min with a microplate reader (Wellreader SK-601; Seikagaku Corporation). Optical density at 405 nm (reference, 492 nm) was read. Values lower than 20 pg/ml were considered negative (12).

Statistical analyses. Wilcoxon's unpaired test was used for analyses of β-glucan levels. $P < 0.05$ was considered significant.

RESULTS

BALFs of mice with PCP. β-Glucan levels of concentrated mouse BALFs are shown in Fig. 1. The levels in all samples obtained from PCP mice were higher than 1,000 pg/ml (mean, 2,631; range, 1,031 to 9,095). In contrast, the levels in control BALFs were lower than 10 pg/ml (mean, 6.5; range, 4.0 to 8.3) despite a vast number of cells from the lung and bronchus. A significant difference between the two groups ($P < 0.001$) was observed.

Human BALFs. *P. carinii* was detected in BALFs from four of eight patients by microscopic examination. Four other patients were diagnosed as follows: two patients had bacterial infections, and cultures of their BALFs and sputa showed *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*; one patient had autopsy-proven lymphoma; and the other patient had interstitial pneumonitis, which responded to zidovudine therapy. β-Glucan levels in the BALFs from patients with PCP ranged from 1,355 to 15,500 pg/ml (Fig. 2). In contrast, BALFs obtained from patients without PCP or pulmonary mycosis showed β-glucan levels of 17 to 615 pg/ml. There was a significant difference ($P < 0.05$) between the two groups. The BALFs of two patients in the group with other lung diseases, one with a bacterial infection and the other with malignant lymphoma, showed high β-glucan levels (302 and 615 pg/ml).

β-Glucan levels in serum. The levels of β-glucan in serum are shown in Fig. 3. All PCP patients except one showed high β-glucan levels (185 to 1,135 pg/ml). One patient with PCP confirmed by microscopic examination of BALF had a low level of β-glucan (8.5 pg/ml). A chest X-ray of this patient had a diffuse reticular shadow, but the patient had few symptoms and no fever or dyspnea. β-Glucan levels in human immunodeficiency virus-positive patients without opportunistic infections were 10 pg/ml or less. Patients with other lung diseases showed low levels of β-glucan in serum. There were significant differences between the PCP and control groups and between the PCP group and the group with other lung diseases ($P < 0.001$).

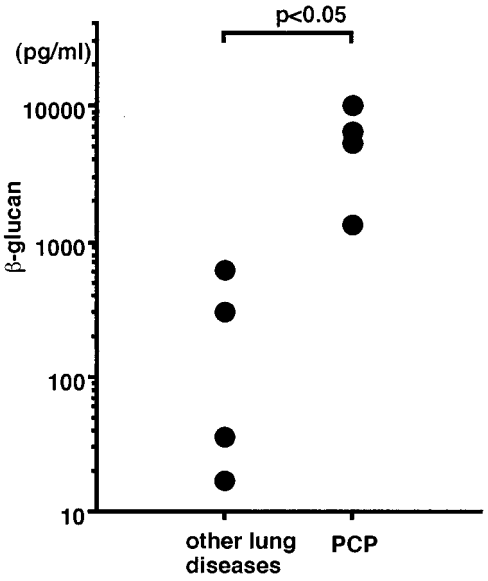

FIG. 2. β-Glucan levels in human BALFs. The *P* value was determined with the unpaired Wilcoxon test.

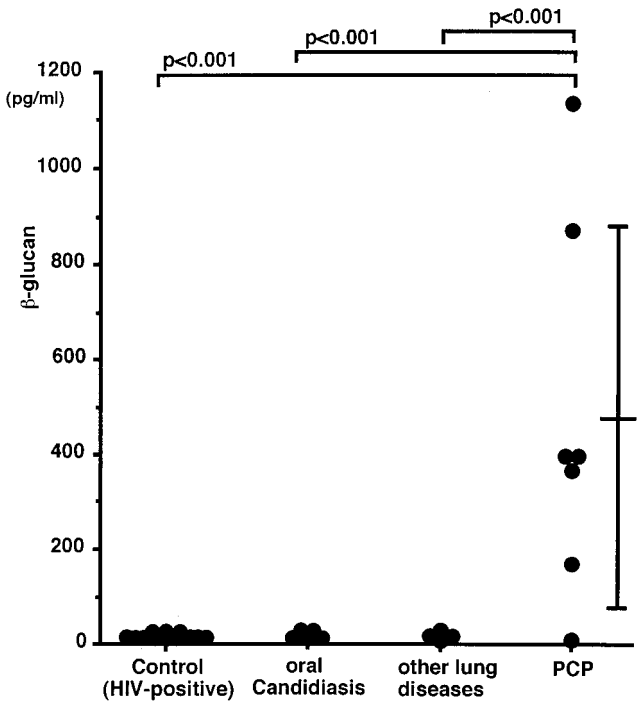

FIG. 3. β-Glucan levels in sera obtained from patients with PCP. *P* values were determined with the unpaired Wilcoxon test.

In five of six patients who recovered from PCP, β-glucan in serum was measured 1 month after the initial measurement. All patients showed a decrease in the β-glucan level to half or less compared with the peak level, along with clinical improvement.

DISCUSSION

Recent genetic investigations revealed that *P. carinii* is closely related to fungi (1). In addition, antifungal agents, like pneumocandin analogs (14) and benanomicin A (16), have antipneumocystis activity. β-Glucan is one of the major components of the yeast cell wall; most fungi have this carbohydrate as well. β-Glucan has been detected in plasma or sera of patients and used for the diagnosis of invasive deep mycosis (12). Moreover, β-glucan detection has been recognized as a simple and sensitive serological marker for fungal infection, although it cannot be used to identify the species of fungi.

Matsumoto et al. (10) first described β-glucan in the *P. carinii* cyst wall. Hoffman et al. (2) showed that *P. carinii* β-glucan-stimulated alveolar macrophages release tumor necrosis factor alpha and speculated that it causes subsequent lung inflammation. β-Glucan synthesis inhibitors such as those in the echinocandin and papulacandin families, developed as antifungal agents, have antipneumocystis activities (14). Therefore, we noticed the possibility that β-glucan detection in serum can be used as a marker of *P. carinii* infection.

There are several published methods for the diagnosis of PCP. One of these methods, PCR, is recognized as a sensitive and specific method for diagnosis (4, 8, 15) and has been used to monitor the disease (13); however, the method is not quantitative if sputum is used as the clinical specimen. In comparison to PCR, β-glucan detection has some advantages despite the lack of specificity for *P. carinii*. First, β-glucan can be measured quantitatively in serum. Repeated checks of its level in serum are useful for evaluation of disease progression and therapeutic efficacy. Second, the procedure used to measure β-glucan is simple and rapid. PCR requires 8 h or more; in contrast, β-glucan detection can be completed in 3 h.

In this study, we used the G test for detection of β-glucan. This assay is a modification of the *Limulus* amebocyte lysate test, which is a sensitive method used to detect endotoxin. Levin et al. (7), who first described the *Limulus* test for endotoxemia, noticed that this test also reacts to fungal infections. Morita et al. (11) found that β-glucan is cross-reactive in the *Limulus* test. Subsequently, several investigations (3, 5, 12) showed that detection of β-glucan is a good marker for deep-seated fungal infections. Our study of BALFs from mice or patients with PCP confirmed that β-glucan is shed from mice and humans infected with *P. carinii* and is detectable by G test.

β-Glucan levels were also elevated in half of the BALFs from patients with other lung diseases. As most fungi have β-glucan, contamination from environmental fungi occurs easily. It is highly possible that the levels of β-glucan in human BALFs, unlike those in BALFs from SPF nude mice, were influenced by contamination through the respiratory tract, medical devices, or containers.

This is the first report of high levels of β-glucan in sera of patients with PCP. Our study revealed that β-glucan was readily detected and quantitated in sera at the time of diagnosis in six of seven patients with PCP. β-Glucan is probably shed from the cyst wall and present in alveolar cavities and may enter the bloodstream through the pulmonary capillary vessel. All follow-up samples showed decreased β-glucan levels after antipneumocystis therapy. Although larger studies are necessary, our study suggests that quantitative detection of β-glucan in serum is a candidate method for monitoring of the course of the disease during treatment and evaluation of drugs developed against *P. carinii.*

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