Evaluation of Sandwich ELISA Galactomannan Test in Samples of Positive LA Test and Positive Aspergillus Antibody

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Objective The detection of circulating *Aspergillus* galactomannan antigen is a useful tool for serodiagnosis of aspergillosis. However, the latex agglutination test for the detection of galactomannan is not completely reliable due to its low sensitivity. The sandwich ELISA was developed to achieve high sensitivity. **Materials** The sandwich immunocapture ELISA was evaluated by testing 56 sero-positive and 56 sero-negative samples of circulating galactomannan detected by LA test retrospectively. **Results** Sixty of the samples were positive for galactomannan as measured by sandwich ELISA. Fifteen samples out of 56 samples negative by LA test were positive by ELISA and 4 samples out of 56 samples positive by LA test were negative by ELISA. Among 47 serum samples positive for anti-*Aspergillus* antibody, 14 samples were positive by ELISA. **Conclusion** In conclusion, galactomannan may be detected in more samples of by the new sandwich ELISA than by LA test.
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Key words: aspergillosis, serodiagnosis, aspergilloma, anti-*Aspergillus* antibody, invasive aspergillosis

Introduction

Aspergillus species are ubiquitous fungi and have been implicated as the causative agents of a variety of lung disorders in humans. These disorders include allergic, saprophytic, and systemic manifestations. The allergic disorders mainly occur in atopic persons, and invasive or systemic diseases occur in immunosuppressed individuals (1). Aspergillosis is a major cause of morbidity and mortality in granulocytopenic patients. With the increasing use of aggressive chemotherapy causing prolonged granulocytopenia in patients with cancer, the risk of disseminated fungal infection is increased. Although *Aspergillus* species are known to be the most common fungal pathogens responsible for disseminated infection, diagnosis of such infection may be difficult (2).

During the past decade, substantial progress has been made in the development of new approaches and methods for the serological diagnosis of the mycoses. Clinically relevant antigens have been adapted for use in immunoassays for the detection of specific antibodies (3), and methods that detect fungal antibody in body fluids have been progressively refined (4). With a growing number of reports on the incidence of aspergillosis and an increase in the number of immunosuppressed

individuals in the population, more rapid methods and more reliable reagents for immunodiagnosis are needed.

Serodiagnosis relies on the detection of circulating antigens produced by *Aspergillus fumigatus* and other *Aspergillus* species. Galactomannan (GM) is one such antigen that is measurable in sera from patients with aspergillosis (5). Most assays to detect antigenemia use latex agglutination but its sensitivity does not appear to be sufficient (6). To improve the sensitivity of GM detection, a double direct sandwich ELISA was designed with the same monoclonal antibody as acceptor and detector, which recognizes the galactofuran side chain of the GM molecule (7).

In this study, we evaluated the usefulness of an enzyme-linked immunosorbant assay (ELISA) for the detection of galactomannan antigen and compared the results with those of latex agglutination. We also assessed the usefulness of the sandwich ELISA test for the detection of circulating galactomannan in anti-*Aspergillus* antibody-positive serum samples.

Materials and Methods

Serum samples

A total of 112 serum samples were obtained from patients

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in Nagasaki University Hospital from 1992 to 1997. Seventy-three samples were from patients with aspergillosis, pulmonary aspergilloma (54 samples), invasive pulmonary aspergillosis (8 samples), chronic necrotizing pulmonary aspergillosis (3 samples), aspergillus pyothorax (3 samples), allergic bronchopulmonary aspergillosis (3 samples), and aspergillus fungemia (2 samples). The diagnosis of pulmonary aspergilloma was made based on the presence of a fungus ball detected on the chest X-ray, a positive result for aspergillus antibody in the serum, and isolation of *Aspergillus* spp. from the sputum or bronchial aspirate. The diagnosis of invasive pulmonary aspergillosis was diagnosed by the histopathological findings for the lung or isolation of *Aspergillus* spp. from the sputum or by the sudden appearance of infiltration shadows in immunosuppressed patients, despite treatment with broad-spectrum antibiotics. The diagnosis of aspergillus pyothorax was made based on the isolation of *Aspergillus* spp. from a sample of pleural effusion. The diagnosis of allergic bronchopulmonary aspergillosis was done according to the criteria reported by Rosenberg et al (8). Chronic necrotizing pulmonary aspergillosis was diagnosed by histopathological findings for the lung in the patients with mild immunosuppression or underlying lung diseases. The diagnosis of aspergillus fungemia was based on the isolation of *Aspergillus* spp. from the blood culture.

Thirty-nine serum samples were from the patients with diagnoses other than aspergillosis, consisting of lung cancer (6 samples), pulmonary fibrosis (5 samples), bacterial pneumonia (5 samples), atypical mycobacteriosis (2 samples), pulmonary cryptococcosis (6 samples) and candidemia (2 samples), and patients with hemospitum (5 samples) and healthy volunteers (8 samples). Sera were kept at 4°C for testing (within 48 hours), then stored at -20°C. The sandwich immunocapture ELISA was evaluated by testing 56 sero-positive and 56 sero-negative samples of circulating galactomannan, as detected by LA test, and 47 antibody-positive serum samples from the 44 patients with pulmonary aspergilloma and 3 patients with allergic bronchopulmonary aspergillosis.

Detection of *Aspergillus galactomannan* antigen

Circulating *Aspergillus* galactomannan was detected using both latex agglutination (LA) test (Pastorex *Aspergillus*, Sanofi Diagnostics Pasteur, France) and a sandwich immunocapture ELISA (Platelia *Aspergillus*, Sanofi Diagnostics Pasteur). For both techniques, immune-complexes were disrupted and proteins were precipitated by adding 100 µl of treatment solution to 300 µl of undiluted serum in a tightly closed Eppendorf tube which was heated to 100°C for 3 minutes, then centrifuged at 10,000g for 10 minutes. The supernatant was used for both tests.

For the LA test, 40 µl of the supernatant of each was transferred onto disposable plastic cards. Ten microliters of the latex reagent was added and mixed with the supernatant using mixing sticks. The results were recorded after 10 minutes of rotation at 120 rpm on a minirotor. A galactomannan control of 75 ng/ml was included with the kit and tested with each batch of serum. Whenever positive results were obtained, two-fold

dilutions were used.

For the ELISA technique, all reagents were provided by the manufacturer. The sensitized microtiter plates were filled with 50 µl of horseradish peroxidase-conjugated monoclonal antibody-galactomannan MAB Eb-A2 as the capture and detector antibody, followed by 50 µl of the pretreated serum supernatant. Plates were incubated for 90 minutes at 37°C. After thorough washings for 5 times, the reaction was allowed to incubate for 30 minutes in darkness with 200 µl of buffer containing *o*-phenylenediamine. The optical density (OD) was read at 450/620 nm. The threshold of the positive sample was estimated to be approximately 1 ng/ml of galactomannan in human serum. The results were calculated by the following ratio for each test serum: I (cut off index)=OD sample/OD threshold serum. The results were estimated as follows; I≥1.5 positive, 1≤I<1.5 gray zone, I<1 negative.

Detection of anti-*Aspergillus* antibody

The detection of anti-*Aspergillus* antibody was performed by the double-gel diffusion assay for the precipitating antibodies to *Aspergillus fumigatus* (*Aspergillus* Immunodiffusion System, Mercia Diagnosis, Surrey, UK). The test was performed according to the procedure recommended by the manufacturer. The semi-quantitative titer of antigen was determined by the presence of lines of precipitin appearing between the wells corresponding to the 20 mg/ml (+) and 2 mg/ml (2+) antigen levels.

Results

A total of 112 samples were indicated in this study for the detection of galactomannan antigen to compare the usefulness of latex agglutination test and the ELISA assay. Fifteen samples (26.8%) were positive by ELISA in the 56 negative samples by LA test. Fifteen samples from the patients with pulmonary aspergilloma (11 samples), chronic necrotizing pulmonary aspergillosis (2 samples) and aspergillus pyothorax (2 samples) were negative by the LA test, but positive by ELISA. Four samples (7.1%) from 3 patients with pulmonary aspergilloma and 1 patient with pulmonary fibrosis were negative by ELISA among 56 positive by LA test. Four samples were estimated to be negative by ELISA in 20 samples showing titers of 1 and 2 in the latex agglutination test. There was no negative sample by ELISA in the sera with the titer>1:4 of LA test (Table 1).

The number of the positive galactomannan antigen in the patients with various different forms of aspergillosis is summarized in Table 2. Among 47 anti-*Aspergillus* antibody positive sera, 14 samples (29.8%) (11 from pulmonary aspergilloma and 3 from chronic necrotizing pulmonary aspergillosis) were positive by ELISA. Nine samples (26.5%) were evaluated as positive in 34 antibody-positive titer as +, and 5 samples (38.5%) were positive in 13 antibody-positive titer as 2+ (Table 2).

Table 1. Detection of Circulating Galactomannan Antigen Using Latex Agglutination and Sandwich ELISA Test

Titer of latex agglutination	Sandwich ELISA ^a			Total
	Positive	Gray zone	Negative	
0	15	13	28	56
1	12	6	2	20
2	17	1	2	20
4	8	0	0	8
8	5	0	0	5
16	3	0	0	3
Total	60	20	32	112

^aThe results were calculated from the following ratio for each test serum: $I = OD \text{ sample} / OD \text{ threshold serum}$. The results were estimated as follows; $I \geq 1.5$ positive, $1 \leq I < 1.5$ gray zone, $I < 1$ negative.

Table 2. Detection of Circulating Galactomannan Antigen Using Sandwich ELISA Test in Antibody-positive Samples

Aspergillus ^a antibody	Sandwich ELISA			Total
	Positive	Gray zone	Negative	
+	9	13	12	34
2+	5	1	7	13
Total	14	14	19	47

^aThe semi-quantitative titer of antigen was determined by the presence of a series precipitin lines appearing at the 20 mg/ml (+) and 2 mg/ml (2+) antigen levels.

Discussion

It is difficult to make an early diagnosis of invasive pulmonary aspergillosis, because the sensitivity of the latex assay for detection of galactomannan antigen does not appear to be sufficient (9). The latex agglutination test yields positive results only during the advanced stages of infection in most patients with suspected invasive pulmonary aspergillosis, and does not contribute to an early diagnosis. A new sensitive double-sandwich enzyme-linked immunosorbent assay can detect less than 1 ng of galactomannan per ml, and can be useful in presumptive diagnosis for the early initiation of antifungal therapy and monitoring treatment in clinically documented invasive pulmonary aspergillosis (10). However, in the present study, four samples of sera which were positive by latex agglutination were

negative by ELISA. This discrepancy could be due to false-positive results by the latex agglutination test, because the results of the latex agglutination test were evaluated subjectively. It was also suggested that there was something inhibiting the reaction of ELISA in these serums. Further studies are needed to clarify this problem.

Pulmonary aspergilloma is typically diagnosed by chest radiography, which shows a fungus ball in the cavity of patients with old pulmonary tuberculosis. More than 80% of the patients show a positive antibody of *Aspergillus fumigatus* as tested by immunodiffusion method. However, mycetoma usually harbors the colonized filamentous fungi, which do not invade the lung tissue. The positive samples tested by ELISA in the antibody-positive sera indicated that some patients with pulmonary aspergilloma shown transient galactomannan antigenemia. This result suggested that *Aspergillus* spp. was not only colonized but possibly invaded the lung tissue. The prognosis of the patients with positive galactomannan tended to be poor based on the results of a retrospective clinical study of the patients with pulmonary aspergilloma (data not shown). A prospective study of the detection of *Aspergillus* galactomannan by ELISA is now under way, and the usefulness of this means of detection for the prediction of the prognosis of patients with pulmonary aspergilloma may be revealed in a future study.

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