JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1991, p. 2363–2367
0095-1137/91/112363-05$02.00/0

Vol. 29, No. 11

Diagnosis of Invasive Candidiasis by Detection of Mannan Antigen by Using the Avidin-Biotin Enzyme Immunoassay

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The diagnosis of invasive candidiasis was attempted by detection of circulating mannan antigen by using an avidin-biotin-amplified enzyme-linked immunosorbent assay (AB-ELISA), and this method was compared with the conventional culture method. Mannan antigen was detected by AB-ELISA in the sera of 16 (84.2%) of the 19 patients with invasive candidiasis. On the other hand, for 34 immunocompromised candidiasis-free patients, including 8 with aspergillosis or cryptococcosis, mannan antigen was positive during only 1 of the 67 febrile episodes and in the serum of none of the 50 outpatients without infections. The results were also negative for all patients with deep-seated mycoses other than candidiasis. However, the mannan level was low (less than 2.0 ng/ml) in the serum of 63.2% of the patients with invasive candidiasis. The positivity rate of blood cultures was 31.6%, and that of blood cultures and/or cultures of samples from sterile sites combined was 47.4%. The advantages of the diagnosis based on antigen detection by AB-ELISA are considered to be a higher sensitivity and elimination of nonspecific reactions by the introduction of the avidin-biotin system and pretreatment of sera by heating. In addition, it is considered essential for high sensitivity that transient mannan antigenemia be determined frequently so that it is not overlooked. In light of its sensitivity and specificity, this method is considered to be clinically useful in the diagnosis of invasive candidiasis.

The increase in the incidence of systemic or invasive candidiasis along with other deep-seated mycoses such as aspergillosis poses a serious clinical problem (10, 18, 23). However, antemortem diagnosis of invasive candidiasis, which is the most frequent of these mycoses, is still very difficult. The true positive rate of conventional blood cultures for *Candida* spp. is low (10, 18), and a significant increase in the anti-*Candida* antibody titer cannot be expected in many hemato-oncological patients because of their underlying conditions (5, 19). Therefore, by those conventional examinations, it is difficult to make an antemortem diagnosis, which would enable administration of appropriate antifungal agents for the prevention of systemic dissemination of *Candida* spp. and cure of the disease.

Various immunological procedures that have evolved as promising methods for specific determination of pathogenic microorganisms in other fields of clinical microbiology have also been applied to the field of clinical mycology. For the diagnosis of invasive candidiasis, these methods are used to detect mannan, glycoproteins, and other protein antigens in the blood of patients, but many of these immunological antigen detection methods have not been proven to be clinically useful. We developed an avidin-biotin-amplified enzyme-linked immunosorbent assay (AB-ELISA) for the detection of *Candida* mannan antigen in the blood. We have already reported the high diagnostic specificity of this method in a study using in which we used murine model of systemic candidiasis (30).

In this study, the usefulness of AB-ELISA for the diagnosis of invasive candidiasis was evaluated in immunocompromised patients, many of whom were children with hemato-oncological disorders, and was compared with the conventional culture method.

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MATERIALS AND METHODS

Patients and controls. The subjects were 19 patients who were diagnosed as having invasive candidiasis between June 1981 and October 1989. The patients were examined for mannan antigen by AB-ELISA. Fifteen patients were treated at the Department of Pediatrics, Chiba University Hospital, or related institutions, and four patients from other institutions were referred to us for examination. One patient who was encountered before 1983, when the AB-ELISA was being developed, was examined retrospectively, but the examination was prospective for the remaining 18 patients. In patients with hemato-oncological disorders, AB-ELISA was carried out two to three times a week; the AB-ELISA was also carried out on samples obtained before febrile episodes. A total of 120 serum samples from these 19 patients were examined by AB-ELISA. Premature infants were excluded from this study.

The diagnosis of invasive candidiasis was made according to (i) histopathological proof by autopsy or biopsy or (ii) isolation of *Candida* spp. by plural blood cultures and/or cultures of specimens from sterile sites. Fourteen patients were diagnosed by criterion i, and the remaining five patients were diagnosed by criterion ii. In this study, candidemia accompanied by intravenous hyperalimentation without neutropenia and infected foci was judged to be transient candidemia and was not included in the group with invasive candidiasis.

The underlying diseases were hemato-oncological disorders in 13 patients, as shown in Table 1. The other underlying diseases were congenital candidiasis of full-term neonates in two patients, postoperative infections of congenital heart diseases in two patients, diffuse idiopathic interstitial pulmonary fibrosis complicated by pulmonary candidiasis after treatment by pulse therapy with methylprednisolone in one patient, and a liver abscess that occurred during

TABLE 1. Underlying diseases of patients with invasive candidiasis

Underlying disease[a]	No. of patients	No of patients who died
Hematology/oncology		
ALL	8	6
APL	1	1
NHL	2	0
FHR	1	1
VAHS	1	0
Others		
Congenital candidiasis	2	1
Postoperative CHD	2	2
PF after pulse therapy	1	1
Liver injury + IVH	1	0
Total	19	12

[a] ALL, acute lymphocytic leukemia; APL, acute promyelocytic leukemia; NHL, non-Hodgkin's lymphoma; FHR, familial histiocytic reticulosis; VAHS, virus-associated hemophagocytic syndrome; CHD, congenital heart disease; PF, diffuse idiopathic interstitial pulmonary fibrosis; IVH, intravenous hyperalimentation.

intravenous hyperalimentation after liver injury in one patient.

For evaluation of the specificity of AB-ELISA, 34 candidiasis-free patients who were immunocompromised primarily because of leukemia and who had febrile episodes and 50 patients without infections who were treated at the outpatient clinic of the Endocrinology Department served as controls. In these immunocompromised hosts, eight patients with deep-seated mycoses other than candidiasis, consisting of seven patients with aspergillosis and one patient with cryptococcosis, were included in the study.

A total of 211 samples were collected during 67 febrile episodes from 34 immunocompromised hosts with granulocytopenia. Among them, 88 and 123 samples were obtained during 30 episodes in which *Candida* spp. could be isolated from pharyngeal swabs, urine, or feces and during 37 episodes in which no *Candida* spp. could be isolated, respectively.

Preparation of anti-mannan IgG and standard mannan antigen. Antisera and *Candida* mannan antigen were prepared and AB-ELISA was carried out by the methods reported previously (30). Briefly, New Zealand White rabbits were immunized intravenously with heat-treated cells of a *Candida albicans* serotype A strain twice a week for 10 weeks, and antisera were collected when the agglutinin titer was 1:1,024. These antisera were further fractionated to immunoglobulin G (IgG) by salting out IgG with ammonium sulfate and DEAE-cellulose column chromatography (DE23; Whatman, Inc., Clifton, N.J.). They were confirmed to precipitate only with *Candida* mannan by the Ouchterlony test.

Purified standard antigen of the mannan of the *Candida* cell wall was prepared by the method of Peat et al. (26) by using *C. albicans* N-7, a serotype A strain derived from a human liver abscess, was provided by H. Taguchi (The Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University). This purified antigen had a protein content of 0.5% of the total weight by the method of Lowry et al. (17) and showed a single peak corresponding to mannose by gas-liquid chromatography.

Conjugation methods. Biotin-specific IgG conjugate was prepared by the method of Nerurkar et al. (24) from biotin succinamide ester (Sigma Chemical Co., St. Louis, Mo.),

TABLE 2. Results of antigen detection by AB-ELISA

Group	No. of patients (no. of serum samples)	
	Total	Positive for mannan
Invasive candidiasis	19 (120)	16 (65)
Control		
Febrile episode group		
Colonized with *Candida* spp.	30 (88)	0 (0)
Not colonized with *Candida* spp.	37 (123)	1 (2)
Infection-free group	50 (50)	0 (0)
Total of controls	117 (261)	1 (2)

and egg avidin-alkaline phosphatase conjugate was prepared by the method of Voller et al. (31) from egg avidin (Sigma) and alkaline phosphatase type VII-S (Sigma).

AB-ELISA. Anti-*Candida*-specific IgG was diluted with sodium bicarbonate buffer (pH 9.6) and was placed in wells of a polystyrene microtiter plate (Immunoplate 1; Nunc, Roskilde, Denmark) and incubated overnight at 4°C. The plate was washed five times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) before use. The same rinsing procedure was repeated after each reaction. The samples, which were pretreated by heating as described below, were placed in wells in triplicate or quadruplicate and were allowed to stand for 2 h at room temperature. Then, biotin-specific IgG conjugate diluted in PBS-T with 1.0% bovine serum albumin was placed in the wells at 37°C for 1 h. After rinsing, egg avidin-alkaline phosphatase diluted in PBS-T was placed in each well at 37°C for 30 min. Then, the substrate (*para*-nitrophenyl phosphate; Sigma), which was dissolved in diethanolamine buffer at 1 mg/ml, was placed in the wells at room temperature for 30 min. Absorbance was determined at 405 nm by using a Dynatech microELISA reader MR 580 (Dynatech), and the mannan concentration was estimated by using the standard curve obtained by simultaneous measurement of the standard antigen. The negative control was measured at five time points, and samples were regarded as positive when the A_{405} of the sample was the mean + 3 standard deviations of the value of the negative control or greater. The sensitivity of this method in heat-treated sera was 1.0 ng/ml.

The heat pretreatment of serum samples was done by the method of Lew et al. (16). The serum sample was diluted with 4 volumes of PBS-T, autoclaved with 1/10 the volume of disodium EDTA at 121°C for 5 min, and centrifuged at 10,000 × *g* for 15 min. The supernatant was used for AB-ELISA.

Determination of sensitivities of AB-ELISA for mannans from various *Candida* species. Interspecies differences in the sensitivity of the AB-ELISA for the detection of mannan antigen were studied by using seven strains of six species of the genus *Candida*: *C. albicans* serotypes A and B, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. glabrata*, and *C. krusei*. These strains were provided by the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University.

Crude mannan antigens were prepared by heat extraction and were used for the determination of the sensitivity of AB-ELISA after equalizing the polysaccharide components, which were determined by the phenol-sulfuric acid method (4), among strains.

Statistical analysis. Statistical analyses were made by Student's *t* test, and $P < 0.05$ was regarded as significant.

RESULTS

As shown in Table 2, *Candida* mannan antigen was detected by AB-ELISA in the sera of 16 of the 19 patients with invasive candidiasis, with a sensitivity of 84.2%. In sample numbers, 65 of 120 serum samples were positive for mannan antigen in these patients.

In the 34 immunocompromised hosts in the control group with febrile episodes, the results were false positive for 2 samples obtained during 1 of the 37 *Candida* colonization-free episodes, i.e., in 1 (1.5%) of 67 episodes in immunocompromised negative controls. In these two false-positive samples, the antigen concentration was 1.0 ng/ml, which was almost the detection limit. The test for the antigen was negative for all 30 patients with febrile episodes colonized with *Candida* spp. and for 50 infection-free outpatients of the Endocrinology Department.

No false-positive results were observed in the eight immunocompromised hosts who had deep-seated mycoses other than candidiasis.

In the control group, a false-positive reaction was observed by AB-ELISA during 1 of the 117 episodes, with a specificity of 99.1%. The positive and negative predictive values and diagnostic efficiency were 94.1, 97.5, and 97.1%, respectively.

Blood cultures were positive for 6 (31.6%) of the 19 patients. Including three positive cultures of samples from sterile sites (bone marrow aspirate, pericardial fluid, and placenta from one patient each), the sensitivity of this examination was only 47.4% (9 of 19 patients). *Candida* spp. were isolated from the blood and samples from sterile sites or at the time of autopsy, as follows: *C. albicans* in nine patients; *C. tropicalis* in two patients; and *C. parapsilosis*, *C. guilliermondii*, and an unidentified *Candida* sp. in one patient each. No significant fungi were isolated from five patients.

In the antigen-positive patients, the peak mannan concentration in serum ranged from 1.0 to 50 ng/ml (10.2 ± 15.0 ng/ml), but it was less than 2.0 ng/ml in the sera of nine (56.3%) of these patients. Overall, the antigen level was less than 2.0 ng/ml in the sera of 12 (63.2%) of the patients with invasive candidiasis.

The peak mannan concentrations in serum were compared according to the results of blood cultures. The mannan concentration seemed to be higher in those with positive than in those with negative blood cultures, but this difference was not statistically significant (22.8 ± 20.2 versus 2.1 ± 2.3 ng/ml; $P > 0.05$). Similarly, no difference in the antigen concentration was observed according to outcome (patients who were cured [4.1 ± 7.3 ng/ml] versus those who died [16.4 ± 20.9 ng/ml]; $P > 0.05$).

In all five patients with positive results both for the mannan assay and blood culture, mannan antigenemia was detected earlier than candidemia (0 to 5 days, 1.8 ± 1.9 days).

Table 3 shows the lower detection limits of the mannans of various *Candida* species by AB-ELISA. Interspecies variations of the detection limit were clearly observed.

In one patient from whom *C. guilliermondii* was isolated, the serum was negative, for antigen, despite positive blood cultures. A concurrence of positive blood cultures and negative antigen levels in serum was observed only in this patient in the present study.

TABLE 3. Lower limits of mannan detection by AB-ELISA against seven strains of *Candida* species

Species	Limit (ng/ml)
C. albicans serotype A	1.0
C. albicans serotype B	2.0
C. tropicalis	1.4
C. parapsilosis	2.8
C. guilliermondii	6.7
C. glabrata	20.0
C. krusei	>50.0

DISCUSSION

In patients with invasive (systemic) candidiasis, there have been attempts to detect *Candida* metabolites and antigens in the serum by means of various biochemical and immunological techniques, including gas-liquid chromatography (14, 22), counterimmunoelectrophoresis (13), hemagglutination inhibition (34), radioimmunoassay (33), ELISA (3, 6, 16, 21, 32), ELISA inhibition (28), and latex agglutination (1, 7, 8, 12, 15, 25). Recently, however, the reports tend to converge on ELISA and latex agglutination. Some of these techniques, e.g., CAND-TEC (Ramco Laboratories, Houston, Tex.), are commercially available, but the clinical reliability of such products still remains controversial (7, 12, 15, 25). Thus, there is practically no established method for immunological detection of *Candida* antigen for the diagnosis of invasive candidiasis.

We developed an AB-ELISA for detection of the *Candida* cell wall mannan antigen in serum to improve the sensitivity, ease of handling, and objectivity of judgment of the immunological diagnosis of candidiasis. The high diagnostic specificity of this method in systemic infections has already been reported in mice. Mannan antigen was shown to be negative at the stage of gastrointestinal candidiasis but to become positive when it developed into systemic candidiasis (30).

In the present study, the sensitivity and specificity of the method were higher than those of the methods reported to date. These favorable results are considered to be derived from the introduction of the avidin-biotin system to ELISA (9, 24) and the pretreatment of samples with heat (16, 21, 27).

Unlike earlier reports of retrospective examinations of patients with pathologically established candidiasis, we studied most of the patients prospectively in the process of their diagnosis and treatment. The three patients with negative AB-ELISA findings in whom systemic candidiasis was established by autopsy were all in the terminal stage of leukemia, and these false-negative results were considered to be due to the fact that few blood samples could be collected and examined.

In one of these patients, *C. guilliermondii* was isolated in blood cultures. As shown in Table 3, the lower detection limit of mannan of this species was 6.7 ng/ml, which was about seven times higher than that of *C. albicans* serotype A. This observation suggests that detection of the antigen of this species and of the antigens of *C. glabrata* and *C. krusei* by AB-ELISA is difficult.

On the contrary, AB-ELISA had a sufficient sensitivity for the mannans of *C. albicans*, *C. tropicalis*, and *C. parapsilosis*, which are frequent pathogens involved in invasive candidiasis.

However, the antigen level was less than 2 ng/ml in the sera of 12 (63.2%) of the 19 patients, including those whose sera were negative for the antigen. This suggests the difficulty of diagnosis on the basis of mannan antigen detection,

as in earlier reports (3, 6, 16). Another difficulty reported by many investigators (2, 6, 11, 16, 34) is that mannan antigenemia in patients with invasive candidiasis is often transient. We attempted to overcome these difficulties by examining samples collected serially from the early stage of the disease. AB-ELISA was performed two to three times a week and was performed on sera obtained before febrile episodes in patients at risk for invasive candidiasis. This is considered to have contributed to the satisfactory results of this study.

Results of the present study indicate that, for diagnosis, AB-ELISA targeted to mannan antigen has the highest sensitivity and specificity among the available methods, despite the disadvantage of the necessity of frequent determinations of antigen levels. On the other hand, the true-positive rate of diagnosis by blood cultures was poor at 31.6%, which is similar to that described in earlier reports (10, 18).

Among the enzyme immunoassay techniques for serum mannan antigen detection for the diagnosis of invasive candidiasis, our AB-ELISA provided the best results because of three improvements. Inhibitors of the antigen-antibody reaction in the serum were removed by pretreatment of samples with heat, the sensitivity was enhanced by introduction of the avidin-biotin system, and the possibility of overlooking transient mannan antigenemia was reduced by serial examination of sera obtained during early stages of disease.

However, a correlation between the antigen concentration and the outcome was not observed statistically. Also, we were unable to determine from our results when antifungal therapy should be discontinued.

Further studies are needed to develop highly sensitive and specific methods for the detection of protein antigens that are considered to be present consistently throughout the course of invasive candidiasis (20, 29) and methods that allow determination of when treatment should be discontinued on the basis of serial determinations of antigen levels.

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