Detection of *Candida* Antigenuria in Disseminated Candidiasis by Immunoblotting

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Received 15 November 1989/Accepted 6 February 1990

Immunoblotting (Western blotting) was used to detect *Candida albicans* antigens in urine of 10 patients with disseminated candidiasis who had two or more positive blood cultures. Twelve urine samples were examined; and antigenuria was found in five of six patients with *C. albicans* infections, in one patient with a mixed *Candida* infection (including *C. albicans*), and in one of two patients with *C. tropicalis* infection. All except one specimen was collected from 2 to 12 days after initiation of amphotericin B therapy. Positive samples showed different numbers of bands in Western blots with an antigen that had an apparent molecular weight of 47,000 in common. This antigen was not found in the urine of patients who had more than 5 days of therapy for candidiasis and who were responding to therapy. The results suggest that Western blotting for *C. albicans* antigens in urine may be a useful method for the diagnosis of disseminated candidiasis and for evaluating antifungal treatment.

The incidence of disseminated candidiasis has increased markedly over the past decade (5, 6, 8, 10, 19–21). The disease has been a common infection among immunocompromised patients and is associated with a mortality of up to 75% (10, 19).

Systemic candidiasis is difficult to diagnose, as there is no typical clinical picture (1, 4, 17). Blood cultures are negative in more than half of the patients with necropsy-proven disseminated candidiasis (4, 12, 15). Various serological procedures have been devised to detect the presence of *Candida* antibodies, ranging from immunodiffusion to more sensitive tests such as counterimmunoelectrophoresis, enzyme-linked immunosorbent assay, and radioimmunoassay (11–13, 21, 22; Editorial, Lancet ii:1373–1374, 1986). Although the accuracy of these tests may be as high as 90% in some groups of patients, in immunocompromised patients, false-positive or false-negative results are encountered (Editorial, Lancet, 1986). Western blotting (WB; immunoblotting) has been used successfully in the detection of antibodies against *Candida* species, but interpretation of *Candida* serology for antibodies is difficult in immunocompromised patients because their B-cell function may be inadequate (7; Editorial, Lancet, 1986). More recently, WB has been used to detect *Candida* antigens in sera of patients with systemic candidiasis, although controversies in interpretation of the results still remain (15, 17, 18; Editorial, Lancet, 1986).

Because of the difficulties cited above and in an attempt to increase our ability to diagnose systemic candidiasis in immunocompromised patients, the experiments reported here were performed to determine whether *Candida albicans* antigens could be detected in urine specimens from patients by WB. *C. albicans* antigen has been demonstrated in sera from patients with systemic disease (16, 24), and if this antigen could also be detected in the urine samples of such patients, it may be an aid to the diagnosis of candidiasis. *C. albicans* B311 (ATCC 32354) was grown on Sabouraud dextrose agar for 18 to 24 h at 25°C. The growth from one slant was inoculated into 50 ml of an amino acid liquid synthetic medium (14) and incubated on a gyratory shaker at 150 rpm at 25°C. After 18 h, a 1-ml sample of this growth was added to 100 ml of the amino acid liquid synthetic medium and rotated at 150 rpm for 24 h at 37°C (23). The cells were harvested by centrifugation at 600 × g for 10 min and washed three times in sterile saline. Organisms grown under these conditions were approximately 90% mycelia and 10% yeast cells, as ascertained by microscopic examination.

A cytoplasmic extract was prepared by a modification of the method of Hazen and Cutler (9). A 50% (vol/vol) suspension of *Candida* cells in normal saline was mixed with an equal volume of glass beads (diameter, 0.45 to 0.50 mm; Biospec, Bartlesville, Okla.) and sonicated with a Branson sonifier (model 185D; Heat Systems, Plainview, N.Y.) over an ice bath for 1 h with 15-min bursts. The sample was then harvested by centrifugation at 1,400 × g for 5 min to remove the cell debris. The resulting supernatant (soluble extract) was mixed with equal parts (vol/vol) of concanavalin A-Sepharose (10 mg/ml; Pharmacia, Uppsala, Sweden), and mannan absorption was conducted for 40 min at room temperature on a shaker. The sample was then centrifuged at 1,400 × g for 10 min, and the sediment was discarded. The supernatant was used as antigen for WB. The protein concentration of the antigen was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Two New Zealand White rabbits (weight, 2.5 kg each) were immunized by subcutaneous injection. The first injections consisted of 1 ml of *C. albicans* cytoplasmic extract mixed with 1 ml of Freund complete adjuvant, and subsequent injections were given weekly with Freund incomplete adjuvant for a period of 4 weeks and then monthly for a period of 2 months. Rabbits were bled from the central ear artery before day 1 and 10 days after the last injection. The rabbit serum which gave the strongest reaction against *C. albicans* cytoplasmic antigen by WB was used in all future tests.

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WB was performed by using the Mini Protean II test system (Bio-Rad Laboratories) (3). Briefly, antigens, serum, or concentrated urine samples were diluted 1:5 in sample buffer with 2-mercaptoethanol and heated for 5 min at 100°C. Pyronin (Bio-Rad Laboratories) was used as the tracking dye, and prestained molecular weight standards (Bio-Rad Laboratories) were included in each day's test. Polyacrylamide gel electrophoresis was conducted with a 10% resolving (lower) and a 3.5% stacking (upper) gel. Each electrophoresis chamber was subjected to 15 mA for 10 min, after which the current was increased to 20 mA for about 50 min or until the dye was 1 cm from the bottom of the gel plates. The separated proteins were electrophoretically transferred to nitrocellulose paper (pore size, 0.45 μm; Bio-Rad Laboratories) for 30 min at 70 V followed by 2.5 h at 100 V. The nitrocellulose sheets were incubated at 4°C overnight in Blotto buffer (5% skim milk) and cut into strips (width, 2 to 3 mm) for the enzyme immunosorbent assay. The strips were incubated at 37°C for 3.5 h with rabbit antiserum to C. albicans or preimmune rabbit serum controls (1:100), washed, and incubated with biotinylated anti-rabbit immunoglobulin G (1:1,000; Vector Laboratories, Burlingame, Calif.) for 30 min. After washing, the strips were incubated with horseradish peroxidase-avidin D (1:1,000; Vector Laboratories) for 30 min and washed. Color was developed with 4-chloro-1-naphthol in the presence of 0.01% hydrogen peroxide.

The sensitivity of WB for C. albicans antigen was ascertained by reacting the cytoplasmic extract in dilutions from 1:10 through 1:2,000 against rabbit antiserum to C. albicans. Ten patients who had two or more positive blood cultures for Candida species at least 24 h apart were selected for testing. Six patients had positive blood cultures for C. albicans, one patient had a positive blood culture for both C. albicans and C. parapsilosis, two patients had positive blood cultures for C. tropicalis, and one patient had a positive blood culture for C. parapsilosis. Random urine samples were collected from these patients. All patients except one were receiving amphotericin B therapy for between 3 and 14 days. All urine samples were cultured for fungi by plating them onto Sabouraud dextrose agar plates. Urine samples from patients with disseminated aspergillosis and healthy male volunteers were used as controls. Urine samples were concentrated approximately 10 times against polyethylene glycol 20,000 (Fisher Scientific Co., Pittsburgh, Pa.) in dialysis tubing with a molecular weight cutoff of 6,000 to 8,000 (Spectra/Por 1; Spectrum Medical, Los Angeles, Calif.). Dialysis tubing containing the urine sample was submerged in polyethylene glycol flakes and removed when the urine volume had decreased about 10-fold.

Our Candida cytoplasmic extract had a protein concentration of 884 μg/ml. WB with the rabbit antiserum to C. albicans could detect this antigen up to a dilution of 1:1,000 (Fig. 1). The limit of antigen detection was, therefore, 0.88 μg of protein.

The C. albicans antigen bands were detected by the rabbit antiserum recognized nine bands in our Candida antigen preparation (Fig. 1). These were seen in the 37- to 88-kDa areas. As the antigen was progressively diluted, the lower-molecular-mass bands disappeared. At 1:200 dilution of the antigen, the 20-kDa band could no longer be detected, and the bands in the 30-kDa area were only faintly visible. The latter bands disappeared at 1:500 dilution of the antigen. Bands in the 37- to 88-kDa areas persisted up to antigen dilutions of 1:1,000, with the strongest bands being in the 37-, 47-, and 88-kDa areas. Preimmune rabbit serum did not react with C. albicans antigen.

Antigen bands were seen in urine samples of five of seven patients with C. albicans-positive blood cultures (Fig. 2). Urine samples from patients 1, 3, and 6 showed three to five bands; urine from patient 2 showed one strong band in the 47-kDa area; urine from patient 5 showed a weak band in the 47-kDa area only. Further concentration of the urine samples did not increase the intensities of these weaker bands. The only other antigen bands in patients 1, 3, and 6 were in the 37-kDa area. Weak bands in the 20-kDa area were seen in the urine samples from patients 3 and 7.

Of the urine samples from patients with Candida infections other than C. albicans, those from patients 8 (C. tropicalis) and 10 (C. parapsilosis) did not show any discernible bands. Patient 9 (C. tropicalis) showed a weak band in the 47-kDa area. No bands were seen in the urine sample from patient 7, a patient whose blood culture grew both C. albicans and C. parapsilosis. The 47-kDa band appeared to be present in all the positive urine samples. No C. albicans antigens were detected in either serum or urine samples from four healthy controls and two patients with disseminated aspergillosis. All urine samples tested were negative for fungi by culture on Sabouraud dextrose agar plates.

FIG. 1. Western blot of C. albicans cytoplasmic antigen (dilutions from 1:10 through 1:1,000) reacted against rabbit antiserum (1:100) to C. albicans. Molecular mass standards for proteins are expressed on the left, in kilodaltons.

FIG. 2. Western blots of urine samples from 10 patients (cases) with disseminated candidiasis reacted against rabbit antiserum (1:100) to C. albicans. The numbers below the strips are the corresponding patient number. Molecular mass standards for proteins are expressed on the left, in kilodaltons.
Urine samples from 10 patients with proven candidiasis were tested by WB for C. albicans antigen (Table 1). Antigen bands were detected in five of seven patients with C. albicans-proven infection (patients 1, 2, 3, 4, and 6). Among these five patients, urine samples were collected from three of them (patients 1, 5, and 6) 3 or 4 days after the start of effective therapy, and urine was collected from one (patient 2) on day 8 of treatment, with a poor response. Urine was collected from patient 3, who did not receive antifungal treatment, on day 3 of his illness. In patient 6, the antigen became negative after the patient had received 10 days of effective therapy. After 10 days of treatment urine was collected from one patient (patient 4) with C. albicans fungemia who showed no antigen in his urine and who was showing a good therapeutic response; hence, antigenuria could be negative on this basis. These findings suggest that antigenuria may be influenced by the length and efficacy of the treatment. Antigenuria has disappeared in humans treated for histoplasmosis and has occurred in those with recurrent disease (25). In another study using a rat model with aspergillosis, antigenemia disappeared after the animals were treated with amphotericin B (21).

In the two patients with C. tropicalis infection (patients 8 and 9), one (patient 9) showed a weak C. albicans antigen band in his urine and serum samples; both of these samples were collected 2 days after the positive blood culture. Since a low cross-reactivity between C. albicans and C. tropicalis has been demonstrated (2, 18) and since C. albicans fails to appear in blood cultures in more than half of the patients with disseminated candidiasis (13, 15), it is possible that this patient had a cross-reacting antigenemia or a combined infection. In the urine sample from patient 7, a patient with both C. albicans and C. parapsilosis infections, antigen bands were very weak. The urine from the patient who had a C. parapsilosis fungemia (patient 10) was negative for the C. albicans antigen. Since cross-reactivity between C. albicans and C. parapsilosis has been found (2), we might expect to find cross-reacting antigens in the urine. However, in this case, the sample was collected 12 days after effective therapy.

The number of antigen bands appears to be proportional to the severity of infection and the presence of renal involvement. The patient who showed the strongest and largest number of bands (Fig. 2, patient [case] 3) was a patient with acquired immune deficiency syndrome (AIDS) who had multiple organ involvement and bilateral multiple renal abscesses caused by C. albicans that were found at the time of autopsy. Additional studies need to be performed to determine whether renal involvement contributes to the presence of C. albicans antigen in the urine. Of the 12 urine samples tested in this study, all had negative fungal cultures. The antigen appearing in the 47-kDa area was the common antigen present in all positive urine tests. This antigen appears to be specific for C. albicans (18) and has been detected in the serum of patients with invasive disease (18).

WB for the detection of C. albicans antigens in urine appears to be a promising method for the diagnosis of disseminated candidiasis, for monitoring the effects of treatment, and for excluding the possibility of relapsing infection. Urine sample collection is not an invasive procedure, and there is no need to treat urine samples chemically or otherwise to dissociate the antigen from immune complexes, a procedure which has been found to be necessary for the detection of certain circulating fungal antigens in serum (25; Editorial, Lancet, 1986).

WB is well known for its specificity. Drawbacks to this test are its complexity for routine laboratories and the fact that it takes 2 days to perform. Some questions still remain, such as when is antigenuria first detectable, what is the incidence of antigenuria in the presence of antigenemia, and when does antigenuria clear during therapy? Urine samples need to be collected from patients at set intervals, and further monthly booster injections given to the rabbits could result in a stronger antiserum which could increase the sensitivity of the test. Future clinical trials should be performed to determine whether there are advantages in performing the WB test with urine rather than serum for the diagnosis of disseminated Candida infection.

We thank Theresa M. Cunningham and Dawn Conover, from the Animal Health Service of Sloan-Kettering Institute, and Mpiko Ntsekhe for expert technical assistance.

**LITERATURE CITED**


