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Candida Antigenemia, as Detected by Passive Hemagglutination Inhibition, in Patients with Disseminated Candidiasis or Candida Colonization

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A passive hemagglutination inhibition assay was studied by using a hyperimmune serum from rabbits immunized with whole yeast cells (*Candida albicans* group A). This technique was effective at detecting small amounts of laboratory-prepared mannan or a whole-cell extract of *C. albicans*. Of 32 patients with documented disseminated candidiasis that were tested, 19 showed evidence of circulating antigen by passive hemagglutination inhibition. Three of these patients showed only partial, rather than complete, inhibition. Among 22 colonized patients, 4 showed partial inhibition, and none of 49 normal controls demonstrated inhibition. All of the sera were tested for antibody by agglutination, immunodiffusion, and passive hemagglutination. This last technique added increased sensitivity, but not specificity, to the standard tests already in use. Fourfold or greater titer rises by passive hemagglutination occurred in fewer than one-third of patients with invasive candidiasis and developed in more than one-half of patients who were colonized and did not require systemic anticandida therapy.

Whether or not a patient has candidiasis requiring treatment is one of the most difficult decisions a clinician faces in infectious diseases (1, 3). Sputum, urine, and even blood may yield the organism on culture without reflecting invasive disease. On the other hand, invasive infection may be unaccompanied by positive cultures. Serological tests for antibody as evidence of invasive candidiasis have been studied widely, but both false-positive and false-negative results can occur, especially in immunosuppressed patients who are at high risk (2). Because of this, attempts have been made to detect circulating antigens, cell constituents, or metabolites as indicators of a candida infection requiring therapy (5-7). A passive hemagglutination inhibition test which has shown promise has been described previously (7). We studied patients who were documented as colonized or invasively infected with *Candida* spp. by using immunodiffusion and agglutination tests, adding to these a passive hemagglutination test to detect antibody and a passive hemagglutination inhibition test to detect circulating antigen.

MATERIALS AND METHODS

Patients. Three groups of patients were evaluated. The first group consisted of patients with disseminated candidiasis involving one or more internal organs. A total of 31 patients had autopsy-proven invasive candidiasis with histological demonstration of the organisms; all had multiple blood cultures, and of these, 22 were positive, 10 with *Candida tropicalis* and 12 with *Candida albicans*. One patient had invasive disease proven at surgery (perinephric abscess with positive stains and cultures for *Torulopsis glabrata*). All of these patients were receiving immunosuppressive, antineoplastic therapy. The second group was composed of 21 patients who were colonized. Colonization was established by multiple positive cultures (other than blood or sterile closed space) in patients who were suspected of having an infection, had negative blood cultures for *Candida* spp., and were not treated with an antifungal agent but recovered from the presumed infection. The positive cultures were from the throat, sputum, urine, and wounds. There was no clinical or histopathological evidence of invasive candida infection. The third group was composed of 49 normal controls. Among them, 15 were obtained from people working in the Infectious Disease Service or microbiology laboratory, and 34 were obtained from a random sample of employees at Memorial Hospital. All of the sera were stored frozen at −20°C, and had been obtained in the first two groups during febrile episodes consistent with candida infection or infection with other organisms. All patients who died had serum taken from at least 10 days before death.

Antigen. Candida antigen was prepared as previously described for the immunodiffusion tests (8). Briefly, *C. albicans* group A was harvested with a swab to scrape colonies from a 72-h culture on Saboraud dextrose agar plates, washed three times in 0.1%

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Formol saline, and suspended in a 30% cell suspension in 0.1% Formol saline. This suspension was sonicated in a Branson cell disruptor in an ice bath for 2 h at 150 W with 15-min bursts. The sonicated material was centrifuged in a Sorvall centrifuge (4°C) at 23,000 × *g* for 60 min. The supernatant material was used as antigen.

Antiserum. New Zealand rabbits weighing 4 to 5 kg were immunized once a week, alternately intravenously or intramuscularly, with a 1% suspension of whole *C. albicans* group A for 2 to 3 months. Titers were determined weekly by agglutination and immunodiffusion. Rabbits were exsanguinated when the agglutination titer reached 1:1,024.

Antibody detection. Three methods of antibody detection were compared. Immunodiffusion and agglutination tests were done as previously described (2, 8). The third method was a passive hemagglutination assay (PHA) with concanavalin A to coat glycoproteins and polysaccharides on sheep erythrocytes (SRBC) (4). SRBC were sensitized with 50 μg of concanavalin A per ml (4). The candida antigen used to coat SRBC was the same as the one used for the immunodiffusion test (8) as briefly described above. Control cells were treated with concanavalin A in the same manner, but no antigen was added. Cells were used at a 0.5% suspension in phosphate-buffered saline with 2% heat-inactivated (56°C for 30 min) fetal calf serum. Since the sensitized SRBC are not stable over 24 h, sensitization and antigen coating were done on the day of each test.

PHA was adapted to a microtiter plate system. All test sera were heat inactivated by incubation at 56°C for 30 min. The sera were absorbed by using 1 part serum (0.2 ml) to 4 parts (0.8 ml) of a 10% suspension of SRBC. The SRBC, however, were packed by centrifugation before addition to avoid dilution of the serum. After incubation for 30 min at room temperature, the sera were tested with uncoated cells to assure absorption of any heterophile antibody. Serial dilutions of 25 μl of test serum were prepared in buffer (phosphate-buffered saline, pH 7.2) with 2% heat-inactivated fetal calf serum. A 25-μl amount of a 0.5% suspension of SRBC previously coated with antigen was added to the serum in each well and allowed to incubate overnight at 4°C. The last well with complete agglutination was considered the endpoint. Uncoated cells were used in control tests.

Antigen detection. A PHA inhibition (PHA-I) test was done as follows. A 25-μl amount of hyperimmune rabbit serum diluted in phosphate-buffered saline with 2% inactivated fetal calf serum to twofold below the maximum agglutination titer was placed in all test wells. Sera to be tested for inhibition (previously heat inactivated and SRBC absorbed) were then serially diluted starting at 1:2 in the hyperimmune rabbit serum. Positive controls were normal human serum which contained candida antigen (diluted 1:32) and another which contained mannan (500 μg/ml), and negative controls were normal human sera. The normal human sera used were chosen because they had no detectable candida antibody by the PHA test. After a 1-h incubation at room temperature, 25 μl of coated SRBC (0.5% suspension) or uncoated control cells was added, and the plates were incubated overnight at 4°C. The inhibition titer was the highest dilution which inhibited agglutination of the antigen-coated SRBC with the rabbit antiserum. The positive control inhibited to a titer of 1:16,834, whereas the negative controls showed no inhibition.

RESULTS

The PHA-I test was effective for detecting small amounts of *C. albicans* group A mannan. As little as 20 ng of laboratory-prepared *C. albicans* group A mannan (kindly supplied to us by Errol Reis, Center for Disease Control, Atlanta, Ga.) per ml could be routinely detected. Yeast extracts (sonicated cell supernatant) of *C. albicans* group A could be detected at a maximum titer of 1:16,384. This system could also detect *C. albicans* group B mannan (also supplied by Errol Reis), but at a concentration of 200 ng/ml.

Table 1 shows the values obtained in the 19 positive patients with disseminated candidiasis, by the PHA-I test. Of the patients, 16 had a positive reaction for antigen, with a maximum titer of 1:64 (range, 1:8 to 1:64).

Partial reactions between complete inhibition of agglutination and complete agglutination were occasionally seen as SRBC buttons which were hazier than the controls. As shown in Table 1, of the 19 patients with disseminated candidiasis and positive tests, 3 had partial reactions. These three patients had higher antibody titers than did the others who showed complete inhibition. The 16 patients who showed complete inhibition by the PHA-I test had no antibody, or very low titers, when tested by agglutination, immunodiffusion, or PHA.

Of 22 patients with invasive candidiasis and

TABLE 1. *Underlying diseases in patients colonized with Candida spp. and with invasive candidiasis*

Disease	No. of patients colonized	No. of patients with invasive candidiasis
Acute leukemia	2	12
Chronic lymphatic leukemia	0	3
Erythroleukemia	1	3
Lymphoma	2	2
Solid tumors	14	6
Others	3[a]	6[b]

[a] Of the three patients colonized, one had agranulocytosis, one had chronic granulomatous disease, and one had encephalitis of unproven etiology.

[b] Of the six patients with invasive candidiasis, three had bone marrow transplants, one had a liver transplant, one had aplastic anemia, and one had Wegener's granulomatosis.

fungemia, 15 showed PHA-I (14 showed complete inhibition), whereas of 10 patients with invasive candidiasis without detected fungemia, 4 were positive (2 showed complete and 2 showed partial inhibition).

Table 1 also shows the results obtained in the 4 colonized patients who had partial inhibition by the PHA-I test. These 4 patients also had high antibody titers.

Antibody titers were low for all controls. PHA titers ranged from less than 1:8 to 1:128, agglutination titers ranged from less than 1:8 to 1:32, and immunodiffusion tests were negative. A rising antibody titer was not found to be useful in making a diagnosis of disseminated candidiasis or of colonization (Table 2). There were seven patients with invasive candidiasis who had fourfold PHA titer decreases when the PHA-I test became positive. Among these same patients, two had fourfold decreases in agglutination titers, and in one, the immunodiffusion reaction became negative. The PHA reaction was a more sensitive test for antibody detection as compared with agglutination or immunodiffusion. Specificity for invasive disease was lacking for all of the antibody tests, by both high titer and rising titers (Table 2). Among the 13 patients with disseminated candidiasis and negative PHA-I reactions, 9 were tested on several occasions, and 4 of these 9 patients (44%) showed fourfold or greater rises in antibody titer by PHA. Among the 18 colonized patients with negative PHA-I reactions, 12 were tested on multiple days, and 7 (58%) showed a significant rise in antibody titer.

The stability of the reactions was tested by repeating PHA-I tests 6 months after the sera were stored at −20°C. Of 26 reactions, 10 were stable, 2 decreased by 3 dilutions, and 14 became negative. There were no tests done between 2 and 6 months after storing the blood.

DISCUSSION

The PHA-I test was effective at detecting small amounts of laboratory-prepared mannan

TABLE 2. *Serological tests in patients with positive PHA-I tests*

Patient no.	Reciprocal of dilution				Organism in blood culture	Time of positive test relative to positive blood culture
	PHA-I[a]	PHA[a]	Direct agglutination[a]	Immunodiffusion		
With disseminated candidiasis						
1	32	2	–	–	*C. tropicalis*	1 Day before
2	64	2	8	–	*C. tropicalis*	5 Days before
3	64	–	–	–	*C. tropicalis*	3 Days before
4	8	–	–	–	*C. albicans*	2 Days before
5	64	4	–	–	*C. tropicalis*	10 Days before
6	16	–	–	–	*C. tropicalis*	14 Days before
7	32	4	8	–	*C. albicans*	3 Days before
8	32	–	–	–	*C. tropicalis*	Same day
9	64	–	–	–	*C. tropicalis*	Same day
10	64	–	16	–	*C. albicans*	1 Day before
11	16	–	8	–	Negative	
12	16	–	32	–	*C. albicans*	1 Day before
13	64	8	NT[b]	NT	Negative	
14	16	–	–	NT	*C. albicans*	14 Days before
15	16	–	NT	NT	*C. albicans*	1 Day before
16	8	–	–	–	*C. albicans*	2 Days before
17	Partial	32	NT	NT	Negative	
18	Partial	128	128	+	Negative	
19	Partial	64	64	+	*C. albicans*	9 Days before
Colonized with *Candida* spp.						
1	Partial	Partial	64	+		
2	Partial	Partial	256	+		
3	Partial	128	64	+		
4	Partial	64	16	–		

[a] PHA-I, PHA, and direct agglutination values are expressed as titers.
[b] NT, Not tested.
[c] No endpoint could be determined in partial reactions.
[d] This patient had invasive disease proven due to *T. glabrata* and colonization with *C. tropicalis*.

TABLE 3. *Summary of serological tests in controls and patients studied*

Type of patient	Total tested	Antigen detection with PHA-I	No. of patients with fourfold or greater rises in antibody titer on repeated testing by:		
			PHA	Agglutination	Immunodiffusion[a]
With disseminated candidiasis	32	19 (3 partial)[b]	7/26	2/21	2/19
Colonized with *Candida* spp.	22	4 (all partial)	8/15	4/12	2/9
Normal controls	49	0	NT[c]	NT	NT

[a] Immunodiffusion tests showed conversions from negative to positive.
[b] Partial reactions which did not show as complete inhibition as did positive controls (see text).
[c] NT, Not tested.

or a whole-cell extract of *C. albicans*. The PHA-I test was also inhibited by sera from patients, with two types of reactions occurring (Table 3): (i) complete inhibition similar to that seen in the positive controls by using a *C. albicans* cell extract as the inhibiting substance and (ii) partial inhibition which was not as complete and clear-cut as the positive controls and which occurred in patients who also had higher passive hemagglutination antibody titers. Antigen-antibody complexes may produce partial inhibition, but this was not documented. If just the complete reactions are considered, then 50% of patients with disseminated invasive candidiasis were positive for circulating antigen, whereas all of the colonized patients and normal controls were negative. If we include the partial reaction as evidence of circulating candida antigen, then 59% of patients with invasive candidiasis were positive, but 4 of 22 patients who were colonized were also positive. In a previous study with human antiserum in this test (7), none of 18 colonized patients were positive for antigen, but one normal blood donor and 2 hospitalized patients without candida infection showed inhibition. Patients who are heavily colonized might circulate antigen in complexes with antibody. The definition of colonization here included the fact that systemic antifungal therapy was not necessary for recovery from a febile syndrome which could not definitely be attributed to *Candida* spp. by the usual means of clinical evaluation (3). It is possible that some of these colonized patients were invasively infected, circulated antigen, and recovered without treatment. If we use this test as an indication for the institution of therapy and include partial inhibition reactions as positive, then we would be missing fewer than half of the patients who had disseminated disease and required treatment and would be treating some patients who did not need it. The test result is available after overnight incubation rather than 1 to 14 days later as with blood cultures for *Candida*. In a previous study with a PHA-I test (7), 4 of 14 patients (29%) with disseminated candidiasis and 2 of 5 patients (40%) with gastrointestinal candidiasis had a detectable PHA-I reaction. The reason for false-negative reactions could be that there was insufficient circulating antigen, antigen was blocked by antibody from the patients, or the anticandida group A antibody does not include determinants for antigens present in some of the organisms infecting the patients. We did not group our isolates, but did find that 7 of 10 patients with *C. tropicalis* infections demonstrated inhibition, and of those with proven *C. albicans*, 8 of 12 were positive by the PHA-I test. It is interesting that a higher percentage of patients who were fungemic had positive reactions by the PHA-I test.

There is no evident reason for the instability of the inhibiting material. If it were only mannan that caused the reaction, that should be stable. This would suggest that some of the reactions were due to antigens other than mannan, which may have been altered by freezing and thawing.

Antibody tests with PHA added increased sensitivity, but not specificity, to the standard immunodiffusion and agglutination tests already in use.

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