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Specific Immunodiffusion Test for Blastomycosis

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A specific immunodiffusion test for blastomycosis has been developed. The test permitted the detection of approximately 80% of 113 proven cases of blastomycosis. Two diagnostically important precipitins designated A and B were frequently recognized in patients with blastomycosis. Routine use of reference sera containing the A and B precipitins in immunodiffusion tests would permit the specific diagnosis of blastomycosis without the need for parallel tests with coccidioidin and histoplasmin.

Numerous clinical and laboratory studies affirm the inadequacy of the currently available serodiagnostic tests for blastomycosis. These deficient tests are the complement fixation (CF) and immunodiffusion (ID) procedures. Their main limitations are inadequate sensitivity and specificity (2, 3, 5, 11, 12, 13). Blastomycosis, like syphilis, tuberculosis, and other systemic mycotic infections, is an imitator of other disease syndromes. The clinical manifestations may be nonspecific and vague or acute and severe. Blastomycosis has no pathognomonical symptoms or radiological features (2, 7). Ideally, the diagnosis of blastomycosis is established by histological detection of the etiologic agent or by its isolation from clinical materials. Unfortunately, these processes take time to carry out or may be negative, to the serious detriment of the patient's welfare. The obvious lack of and immediate need for a specific serological test for the diagnosis of blastomycosis was ably stated in a recent editorial by Chick (6).

In 1961, Abernathy and Heiner (1) attempted to compensate for the nonspecificity and cross-reactivity of CF tests by using an immunodiffusion test. These workers found 64% or 14 of 22 patients with blastomycosis to have precipitins to a mycelial filtrate of *Blastomyces dermatitidis*. They reported that the ID test was as sensitive as their CF test. In addition, the studies of Abernathy and Heiner (1) revealed that precipitins to blastomyces antigens could be found in some patients with coccidioidomycosis or histoplasmosis, but that these could be proven to be cross-reactions by simultaneously testing the sera with antigens to *B. dermatitidis*, *Coccidioides immitis*, and *Histoplasma capsulatum*.

Busey (3) reported that less than 50% of the proven cases of blastomycosis reacted in the CF test and that positive reactions occurred with sera from patients with diseases other than blastomycosis. Furthermore, he found that the height of the CF titers could not be relied upon diagnostically, since heterologous antigens often elicited higher titers than the homologous ones. In an attempt to improve the serodiagnosis of blastomycosis, Busey and Hinton (5) developed a micro-ID test with phenolized mycelial (17 to 33 weeks of growth) and yeast-form filtrate (72-h growth) antigens of *B. dermatitidis*. In a study of 80 proven blastomycosis cases, 71 or 89% had sera with precipitins to either or both antigens. Sixty-four or 80% reacted with the yeast antigens, and 38 or 48% reacted with the mycelial antigen. Unfortunately, as with the CF test antigens, both of the ID antigens cross-reacted with sera from patients with other fungal diseases, particularly histoplasmosis.

The continuing need for a specific serological test prompted us to further evaluate the value of yeast and mycelial *B. dermatitidis* antigens in the CF and ID tests for blastomycosis.

MATERIALS AND METHODS

Fungus cultures. Antigens were prepared from yeast or mycelial-form cultures of *B. dermatitidis* strains A-295 and A-373.

Antigen preparation. Yeast-form cellular and filtrate antigens were prepared from 1-week-old brain heart infusion (Difco) broth cultures shaken at 150 to 160 rpm and maintained at 37 C. The cultures were killed by adding Merthiolate to a concentration of 1:10,000. After appropriate sterility checks, the yeast cells were removed by filtration through a Buchner funnel with Whatman no. 1 filter paper.

The cells were washed three times in merthiolated saline, broken, and standardized for use in the CF test (8).

The mycelial cultures of *B. dermatitidis* were grown in Smith's asparagine medium for 6 months according to a protocol described for histoplasmin production (8). The Merthiolate-killed culture was filtered as described above. The mycelial mat was centrifuged for 30 min at 1,100 × *g*, washed once, resuspended in four volumes of merthiolated saline, and broken up with a Sorvall Omni-mixer until approximately 90% breakage occured. Breakage was estimated by microscopic examination.

The aforementioned filtrates of the yeast and mycelial cultures were treated with two volumes of cold acetone and allowed to sit at 4 C for 24 h. The precipitates were separated by centrifugation in the cold at 4,000 × *g* for 20 min. The precipitate was dissolved in a volume of phosphate-buffered saline, pH 7.2, equal to one-tenth of the original filtrate volume. Merthiolate was added as a preservative, and the × 10 concentrated antigens were stored at 4 C. Antigenically similar preparations could be made by concentrating the filtrates with an Amicon PM 10 membrane (Amicon Corp., Lexington, Mass.).

The optimal dilution for each antigen was determined by box titration against sera from humans with blastomycosis. The optimal ID antigen dilution was the highest dilution giving the greatest number of well-defined bands upon reactions with various dilutions of the sera.

CF test. CF tests were performed by the standardized Center for Disease Control Laboratory Branch complement fixation test (14).

ID tests. A modification of Busey and Hinton's micro-ID test was used (4). Melted agar (6.5 ml) was pipetted into a sterile 100 by 15 mm plastic petri dish. After solidification, an additional 3.5 ml of melted agar was poured as an overlay, and a plastic matrix containing 17 seven-well patterns (L. L. Pellet Co., Dallas, Texas) was immediately placed on the liquid agar. Control and unknown sera were placed in the peripheral wells and preincubated for 45 min at 37 C. Antigens were then added to the center well. Each plate was incubated in a moist chamber at 37 C for 48 h, the matrix was removed, and the plate was examined for the presence of precipitins.

Sera. Specimens received in the Fungus Immunology Section of the Center for Disease Control, Mycology Branch, were from patients with culturally proven cases of systemic disease due to mycotic and bacterial agents. Case histories were obtained from the attending physicians. Diagnosis was based upon either cultural or histological studies, or both. All sera were preserved with Merthiolate (0.01%).

RESULTS

Preliminary CF and ID tests were performed with a suspension of homogenized *B. dermatitidis* yeast-form cells, yeast filtrates, homogenized mycelium, and mycelial-filtrate antigens of two isolates of *B. dermatitidis* (A-373 and A-295). These tests indicated that only the homogenized yeast antigen demonstrated sufficient antigenic potency and sensitivity for further evaluation in CF tests. The yeast filtrate antigens were similarly the most satisfactory for ID tests. Essentially there was no difference between the antigens derived from the two isolates studied. Consequently, all of the following results are based upon our findings with one of the cultures (A-373).

Table 1 shows the results obtained with the homologous and heterologous sera with the optimally diluted (1:32) broken yeast suspension used as a CF antigen and the optimally diluted (1:4) ×10 concentrated yeast filtrate used in the ID test. The results show that the ID test was more sensitive than the CF test for detecting blastomycosis cases and that the yeast precipitinogens cross-reacted extensively with heterologous sera, mainly those from cases of coccidioidomycosis, histoplasmosis, and paracoccidioidomycosis.

Attempts were made to distinguish and identify the precipitin bands noted when the *B. dermatitidis* antigens reacted with sera from patients with blastomycosis, coccidioidomycosis, histoplasmosis, and paracoccidioidomycosis. We found that the concentrated yeast filtrate antigens contained three specific diagnostically useful precipitinogens and two nonspecific antigens. In reactions with blastomycosis case sera, the yeast filtrate antigen frequently gave rise to one or two bands. One, which was close to the antigen well, was designated as A, and the second, close to the serum well, was designated B (Fig. 1). A third diffuse band located between A and B bands was encountered with only 1 of 49 case sera. This

TABLE 1. *Reactivity of Blastomyces dermatitidis A-373 yeast antigens in CF and ID tests with 49 homologous and 104 heterologous human case sera*

Disease	No. of sera	No. of positive tests (%)	
		CF[a]	ID[b]
Blastomycosis	49	20 (41)	29 (59)
Coccidioidomycosis	37	18 (48)	4 (11)
Histoplasmosis	47	37 (77)	16 (34)
Paracoccidioidomycosis	3	2 (67)	1 (33)
Actinomycosis	2	0 (0)	0 (0)
Candidiasis	2	0 (0)	0 (0)
Cryptococcosis	9	0 (0)	0 (0)
Nocardiosis	2	0 (0)	1 (50)
Sporotrichosis	2	0[c] (0)	0 (0)

[a] Antigen used consisted of broken yeast cells.
[b] Antigen used consisted of yeast culture filtrate.
[c] One serum anticomplementary.

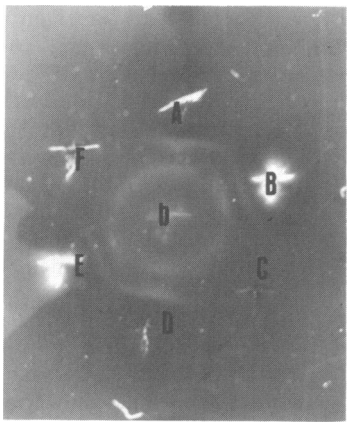

FIG. 1. *Reactions of identity with reference serum containing A and B antibodies (wells A and D) and sera from persons with culturally proven blastomycosis (wells B, C, E, and F). Well b contains yeast culture filtrate antigen.*

serum was obtained from a patient with blastomycosis. A fourth and a fifth band, reactive only with heterologous sera, were also noted. These bands were usually found near the serum well, and one of them probably represents the "c" or common factor recognized earlier by Abernathy and Heiner (1). Experiments designed to relate the A and B lines to the histoplasmosis H and M precipitin bands described by Heiner (9) and to the coccidioidin bands described by Huppert and Bailey (10) showed them to be distinct and characteristic for *B. dermatitidis* only.

Using the micro-immunodiffusion template described by Busey and Hinton (4), we retested all of the sera listed in Table 1. Sera, containing the A and B precipitins, from patients with proven blastomycosis were used as references in the upper and lower central wells. Only sera that produced lines of identity with either the A, or the B, or both reference bands were considered to be positive for blastomycosis. Twenty-eight of the 49 or 57% of the sera from blastomycosis cases were positive. In contrast, all of the heterologous sera were negative.

Table 2 shows the results of CF and ID tests on sera from 113 proven cases of blastomycosis. Sixty-four or 57% of the sera reacted in the CF test. Of these, 54 were ID positive, and 35 or 55% of them showed only A bands. Nineteen showed A and B bands. The B band never occurred alone, but was associated with the A band. Of the 45 CF negative and 4 anticomplementary sera, 35 were ID positive, with 31 or 63% showing the most frequently observed A band. The ID test alone detected 89 or 79% of the cases. Simultaneous use of the two procedures permitted detection of 88% of the cases.

Although serology is valuable in establishing a diagnosis it has limited value in determining the prognosis of a given case. It is evident from the data in Table 3 that one month after treatment with Amphotericin B or stilbamidine none of the patients studied demonstrated significant changes in CF titer or in precipitin numbers. Two of the patients who had recovered from the infection had sera which produced weaker bands or fewer bands than their initial serum specimens.

Those patients who were serologically positive at the start of therapy were also positive one month after therapy, regardless of whether the treatment was successful.

DISCUSSION

A specific ID test having a sensitivity of approximately 80% has been developed for blastomycosis. Our data are in agreement with those of Busey and Hinton (5) that the ID test is

TABLE 2. *Complement fixation and immunodiffusion reactions of 113 proven blastomycosis case sera*

CF Test	ID Test			Total
	+		−	
	A band	A and B bands		
+	35	19	10	64
−	31	4	14	49[a]
Total	66	23	24	113

[a] Four sera anticomplementary.

TABLE 3. *Complement fixation and immunodiffusion test results of sera from selected cases of blastomycosis prior to and after treatment*

Patient	CF Titer				ID Reaction[a]			
	Prior to therapy	Months after therapy			Prior to therapy	Months after therapy		
		1	6	12		1	6	12
M.S.	0	0	0		+	W+[b]	−	
V.P.	1:16	1:8		0	++	+[b]		−
D.H.	1:16	1:32			+	++		
H.T.	1:8	1:8		1:8	+	W+		−
L.H.	1:8	1:8	0		++	++[b]	−	
R.E.	1:16	1:8	0		+	+[b]	−	
A.F.	0	0			++	++[b]		

[a] Symbols: A band, +; A and B band, ++; weak, W; no band, −.

[b] Clinically recovered from blastomycosis.

more sensitive than the CF test with *B. dermatitidis* antigens and that the yeast precipitinogens will cross-react extensively with sera from patients with other systemic fungus diseases. The proper selection and use of reference sera, however, made possible the specific detection of *B. dermatitidis* precipitins without the need to carry out additional or simultaneous tests with coccidioidin and histoplasmin ID antigens.

Our data, like those of Busey and Hinton (5), indicate that the yeast-form antigen of *B. dermatitidis* is the most sensitive for use in the ID test. The increased specificity of our test is attributed to the use of a concentrated filtrate prepared from a 1-week-old, yeast-form culture and the routine use of reference sera that contain A and B precipitins. The production of one or more lines of identity by a patient's serum interacting with antigen constitutes a positive reaction. Sera producing precipitin bands not identical with the specific reference lines are considered suspicious and warrant further study with other fungal antigens.

Some blastomycosis case sera are not easily diagnosed by currently available serologic procedures. These are sera that are CF and ID negative or are CF positive but ID negative. Patients with sera in these categories should be studied intensively for cultural or histological evidence of blastomycosis. In addition, serial serum specimens should be drawn from them at 3-week intervals and examined by CF and ID tests with *B. dermatitidis*, *C. immitis*, and *H. capsulatum* antigens to detect either the appearance of CF antibodies, significant changes in homologous titer levels, or the development of precipitin bands diagnostic for blastomycosis, coccidioidomycosis, and histoplasmosis.

In established cases of blastomycosis, a decline in CF titer or the disappearance of precipitin lines are evidence of a favorable prognosis. The serological response, however, is often not as rapid as the clinical response. Patients who are serologically positive may be monitored for changes in CF titer or precipitins. We have noted more frequent changes with precipitin lines than with CF titers; however, as Abernathy noted (personal communication, 1965), recovery may occur without concomitant disappearance of precipitins. In contrast to Busey and Hinton (5), we observed patients in whom therapy was effective but in whose sera precipitins were still demonstrable 30 days or longer after completion of therapy. In cases where precipitin lines disappeared, these changes occurred 2 or more months after cessation of chemotherapy and after the radiologically detected symptoms cleared and *B. dermatitidis* was no longer isolated.

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