

## Preparation of Reference Antisera for Laboratory Diagnosis of Blastomycosis

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Antiserum was prepared in rabbits against the A antigen and a new antigen (labeled the D antigen) of yeast-phase cells of *Blastomyces dermatitidis* by using immunoelectrophoretic precipitin arcs as vaccine. The antiserum can be used as a reference serum in the immunodiffusion test for identifying antibodies to the A antigen in sera from patients thought to have blastomycosis. Antibodies to the D antigen have not yet been found in human sera. The rabbit antiserum that was absorbed with *Histoplasma capsulatum* yeast-phase cells did not react in the complement fixation test with either *H. capsulatum* yeast-phase or mycelial-phase complement fixation antigen. Therefore, it could also be used as a positive control serum in the complement fixation test. A fluorescent-antibody conjugate prepared from the absorbed serum was found to be valuable for the identification of yeast-phase cells of *B. dermatitidis*.

The immunodiffusion (ID) test is specific for laboratory diagnosis of blastomycosis. Kaufman et al. (11) used a modification of the ID test originally described by Abernathy and Heiner (1) and later by Busey and Hinton (2) and reported the presence of two diagnostically specific precipitin lines (labeled A and B) in 80% of the sera from patients with blastomycosis. A concentrated shake culture filtrate of the yeast phase of *Blastomyces dermatitidis* was used as the antigen in these tests. The B line never occurred alone but was always associated with the A line (11). In many of the sera tested, two additional precipitin lines formed that also reacted with ID antigens of *Histoplasma capsulatum* and *Coccidioides immitis* and were assumed to represent the "c" or common antigen reported by Abernathy and Heiner (1).

Huppert (8) and Gross et al. (5) have shown the importance of using standardized antigens and reference antisera in the ID test to ensure that its results can be properly interpreted. Thus, concentrations of the two reagents must be such that the resulting precipitin lines are sharp and, if applicable, are sufficiently well separated that one can tell whether the precipitin lines formed by the serum of the patient form lines of identity with the reference lines. In the past, reference antisera used in the ID test for blastomycosis have been obtained from patients with culturally proven cases of the disease. The difficulty of obtaining adequate amounts of satisfactory human sera has been the greatest de-

terrent to using the ID test in diagnostic laboratories. If antisera specific for the desired precipitinogen were prepared in animals, this problem would be solved. However, Kaplan and Kaufman (9) reported that antisera prepared with killed intact yeast-phase cultures contained high levels of heterologous antibodies which required three to four absorptions to remove. The latter antisera were used to prepare fluorescent-antibody (FA) conjugates; before absorption these conjugates stained 17 different fungi at intensities ranging from 1+ to 4+.

Recently we were able to prepare specific antisera to the H and M antigens of *H. capsulatum* by separating these two antigens from one another and from other cellular components by immunoelectrophoresis (IE), with the separated precipitin lines used as vaccines (4). We used this same technique to prepare an antiserum against the A antigen and a new antigen we labeled antigen D of *B. dermatitidis*. This antiserum, which required one absorption with yeast cells of *H. capsulatum*, gave a sharp precipitin line with crude homologous ID antigens but did not react with crude ID antigens of *H. capsulatum* or *C. immitis*. Also, an FA conjugate prepared from this antiserum was satisfactory for use in the direct FA test. Thus, an antiserum has been prepared in animals that can be used as a reference serum in the ID test for blastomycosis and can also be used in preparing a FA conjugate for identifying *B. dermatitidis* yeast-phase cells.

## MATERIALS AND METHODS

**Antigen preparation.** All *B. dermatitidis* antigens were prepared with yeast-phase cells of strain A373. The control antigen for the ID test was prepared as recommended by Kaufman et al. (11). It consists of a 10-fold concentrate of a shake culture grown in brain heart infusion broth at 37°C. The complement fixation (CF) antigen was a broken cell suspension of yeast-phase cells grown for 7 days on brain heart infusion agar at 37°C (6).

Four experimental antigens were prepared for immunizing animals.

(i) **Water-soluble antigen.** This antigen was prepared with yeast-phase cells grown on brain heart infusion agar, harvested with saline containing 0.01% thimerosal, and adjusted to a cell concentration of 2% (vol/vol). The cell suspension was stored for 1 week at room temperature, the cells were removed by centrifugation, and the supernatant was concentrated approximately 50-fold with dry carboxymethylcellulose. After this antigen was dialyzed against distilled water, it was used in the ID and IE tests. (For IE experiments it was further concentrated 10 times.)

(ii) **Cell wall antigen.** Thimerosal-killed cells were adjusted to a concentration of 20% (vol/vol) in saline; 50 ml of this suspension was mixed with 25 ml of glass beads (200  $\mu$ m) in a 125-ml wheaton bottle. The mixture was shaken at 4°C for 1 h at 800 rpm in the shaker head of an International PR2 centrifuge. The broken cell suspension was centrifuged for 10 min at 600  $\times$  g, and the supernatant was decanted and saved. The sediment was resuspended in 50 ml of saline, shaken again for 10 min at 800 rpm, and centrifuged at 600  $\times$  g for 10 min, and the two 600- $\times$ -g supernatants were pooled. The sediment was resuspended in saline, the glass beads were allowed to settle out, and the cell walls and cellular debris were decanted. The cellular suspension was washed five times for 10 min each with saline by centrifugation at 600  $\times$  g.

(iii) **Cytoplasmic particle antigen.** The 600- $\times$ -g supernatant from (ii) above was centrifuged at 40,000  $\times$  g and 4°C for 30 min, the supernatant was decanted,

and the sediment was washed four times with saline and resuspended with saline to a volume two times that of the original 20% cell suspension.

(iv) **Cytoplasmic soluble antigen.** The volume of the soluble cytoplasmic material from (iii) above was adjusted to two times that of the original 20% cell suspension.

**Positive control human sera.** Sera from patients with proven cases of blastomycosis and containing antibodies to the A antigen of *B. dermatitidis* were obtained from the Serum Bank, Center for Disease Control. Sera containing antibodies to the B antigen were not available.

**Preparation of antisera.** *B. dermatitidis* antisera were prepared in rabbits, with the four experimental antigens described above used as vaccines (Table 1). Samples of the cell wall, cytoplasmic particle, and cytoplasmic soluble antigens were mixed with equal volumes of complete Freund adjuvant to form a stable emulsion. Rabbits were inoculated with 1 ml of vaccine intradermally in four to six sites on the back and with 0.25 ml of vaccine in each front footpad. After 7 days each animal was given 0.5 ml of vaccine intradermally in two sites on the back. After a 4-week rest period, each animal received 0.3 ml of the original antigen without adjuvant intravenously. Test samples were taken at 7- to 10-day intervals after the last inoculation.

The water-soluble antigen of *B. dermatitidis* was also used to prepare antisera in rabbits. The A and D antigens were separated from other antigens with IE, and the resulting precipitin arcs were used as vaccines. The procedure was essentially that described for preparing antisera to the H and M antigens of *H. capsulatum* (4). Precipitin arcs for the A and D antigens of *B. dermatitidis* were developed with antisera prepared in rabbits immunized with the cell wall antigen.

The vaccines were prepared by pooling the precipitin arcs from several IE runs. When enough arcs had been collected to immunize the desired number of rabbits, they were washed five times with phosphate-buffered saline, pH 7.2, over a period of several days and finally were macerated by being forced through a 21-gauge needle several times (4). This antigen-anti-

TABLE 1. Antibodies observed by ID in antisera from rabbits immunized with *B. dermatitidis* antigens

| Antiserum lot produced | Antigen              | Antiserum lot | Precipitin lines <sup>b</sup> |   |   |              |
|------------------------|----------------------|---------------|-------------------------------|---|---|--------------|
|                        |                      |               | A                             | D | c | Unidentified |
| 1A                     | Cell wall            | None          | +                             | + | w | —            |
| 1B                     | Cytoplasmic soluble  | None          | w                             | w | + | +            |
| 1C                     | Cytoplasmic particle | None          | —                             | — | + | —            |
| 2A <sup>d</sup>        | WSA IE A arc         | 1A            | +                             | — | w | —            |
| 2B <sup>d</sup>        | WSA IE D arc         | 1A            | —                             | + | w | —            |
| 3                      | WSA IE A arc         | 1A            | +                             | — | w | —            |
| 4                      | WSA IE A arc         | 3             | +                             | + | w | —            |

<sup>a</sup> Cellular fractions and IE precipitin arcs were mixed with equal volumes of complete Freund adjuvant and inoculated intradermally and into front footpads. Booster inoculations given intravenously without adjuvant. WSA, Water-soluble antigen.

<sup>b</sup> A, to A antigen; D, to D antigen; c, to c antigen; +, strong precipitin line; w, weak precipitin line; —, no line.

<sup>c</sup> The number in parentheses indicates that two lines were present.

<sup>d</sup> The A or D precipitin arc of water-soluble antigens developed with lot 1A antisera by IE.

body-agar complex was mixed with an equal volume of complete Freund adjuvant until a stable emulsion was formed. The number of precipitin arcs in each pool varied from 24 to 60. For the primary immunization, three-fourths of the vaccine dose for each rabbit was given intradermally in several sites on the back, and the remainder was divided into aliquots and injected into the front footpads. The animals rested for 6 weeks before intravenous booster inoculations of 0.3 ml of the control ID *B. dermatitidis* antigen without adjuvant were given. The intravenous booster inoculation for lot 4 *B. dermatitidis* antiserum consisted of 30 macerated precipitin arcs without adjuvant per rabbit rather than the soluble crude antigen. Test blood samples were taken at 7- to 10-day intervals after the last inoculation.

**Absorption of antisera.** Absorption studies were done on the lot 4 *B. dermatitidis* antiserum. Cell wall antigen and cytoplasmic particle antigen from yeast-phase *B. dermatitidis* and whole cells of yeast-phase *H. capsulatum* were used as absorbing antigens. *H. capsulatum* cells were grown on brain heart infusion agar for 7 days at 37°C, killed with 0.01% thimerosal, and washed three times with saline. The ratio of absorbing antigen to serum was equivalent to a 1:1 ratio of whole cells to serum. Absorption was effected by incubating the mixture at 48°C for 2 h.

**Serological tests.** IE and ID tests were performed as previously described (4). The CF test was the Center for Disease Control Laboratory Branch CF test (14).

**Preparation of FA conjugate.** The immunoglobulin G fraction of antiserum was obtained by diethylaminoethyl column chromatography and labeled with fluorescein isothiocyanate. The unreacted dye was removed by gel filtration on Sephadex G-50.

## RESULTS

**Preparation of antisera.** The responses of rabbits immunized with the different *B. dermatitidis* antigens are shown in Table 1. Antisera against the cell wall antigen (lot 1A) gave two strong precipitin lines and one weak one in ID and IE tests when they were reacted against the water-soluble ID antigen. In the ID test, one of the strong precipitin lines formed a line of identity with the A precipitin line of a human serum known to contain antibodies to the A antigen of *B. dermatitidis* (Fig. 1). The second strong precipitin line formed a line of nonidentity with the reference A precipitin line (Fig. 1). We did not have a reference antiserum containing antibodies to the B antigen and therefore could not determine whether the non-A line was the same as the B line described by Kaufman et al. (12). For this reason we labeled it the D line. When this antiserum was reacted with the ID water-soluble antigen that had been heated for 5 min in a boiling water bath, the A precipitin line was still present, but the D line was not (Fig. 1). Therefore, the A antigen is considered heat sta-

ble, and the D antigen is considered heat labile. In IE with water-soluble antigen in the well and lot 1A antiserum in the troughs, two well-separated strong precipitin arcs and one weak precipitin arc were obtained (Fig. 2). Subsequently the strong arc closest to the anode was identified as the A antigen with the positive control human serum, and the more slowly moving arc was identified as the D antigen with rabbit lot 1A serum (Fig. 3). In IE, the third weak precipitin arc appeared near the antiserum trough as a streak going from the antigen well toward the anode. This line was comparable to the nonspecific c antigen line identified by Heiner (7) in ID antigens of *H. capsulatum* in that it formed lines of identity with ID antigens of *H. capsulatum* and *C. immitis*.

Antisera from rabbits inoculated with cytoplasmic particle antigen lot 1C formed at least one strong and two or more weak precipitin lines in ID and IE tests. These antibodies formed lines of nonidentity with the A and D antigens in ID tests. In IE, the strong precipitin line

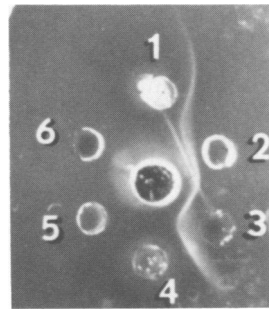


FIG. 1. ID test with lot 1A antiserum prepared with cell walls of *B. dermatitidis*. Center well and well 4, lot 1A antiserum; well 1, human serum containing antibodies to the A antigen; well 2, water-soluble *B. dermatitidis* antigen; well 3, water-soluble *B. dermatitidis* antigen heated in a boiling water bath for 5 min. Wells 5 and 6 were empty. The precipitin line between the center well and well 2 is the D line; the other line is the A line.

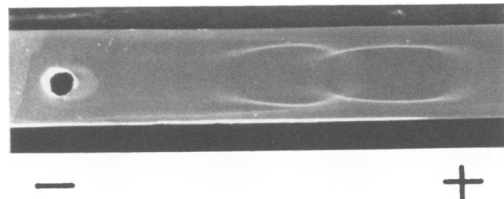


FIG. 2. Precipitin lines formed by lot 1A antiserum on IE slides. Water-soluble antigen electrophoresed from the well; lot 1A antiserum was in each trough. The precipitin line nearest the anode is the A line; the D line is nearest the cathode.

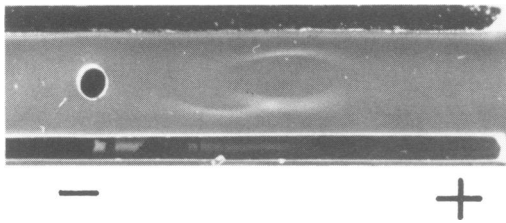


FIG. 3. Identification of A and D antigens of *B. dermatitidis* with IE. Water-soluble antigen electrophoresed from the well. Upper trough, human serum containing antibodies to the A antigen; lower trough, lot 1A antiserum.

developed near the antigen well, with streaking along the antiserum trough. In the ID test, the strong precipitin line formed lines of identity with heat-stable ID antigens of *B. dermatitidis*, *H. capsulatum*, and *C. immitis* (Fig. 4). The heat stability and nonspecificity of these antigens were comparable to those of the c antigen which Heiner (7) identified in *H. capsulatum*, *B. dermatitidis*, and *C. immitis* and therefore were called the c antigens and antibodies. The c antigens of *B. dermatitidis* consist of two or more nonspecific antigens.

In the ID test, antisera from rabbits inoculated with cytoplasmic soluble antigen lot 1B contained antibodies that reacted only with the c antigens of *B. dermatitidis*, *H. capsulatum*, and *C. immitis*.

Precipitin arc vaccines were prepared by electrophoresing the water-soluble antigen and developing the separated A and D antigens with lot 1A antiserum prepared from cell walls. Antiserum lots 2A and 3 (Table 1) were prepared with the A precipitin arcs, and lot 2B was prepared with the D precipitin arcs. Each of the antisera reacted with the antigen present in the precipitin arcs used as vaccines to form strong precipitin lines and formed weak precipitin lines with the c antigen. Only one-third of the rabbits responded to the A antigen when a pool of 24 to 29 precipitin arcs per rabbit was used as the primary inoculum; all of the rabbits inoculated with 30 precipitin arcs of the D antigen responded. An additional lot of antiserum to the A antigen (lot 4) was prepared with a pool of 60 precipitin arcs plus adjuvant as the primary inoculum and a pool of 30 precipitin arcs without adjuvant as the booster inoculum. The precipitin lines were developed with lot 3 antiserum. All of the rabbits responded to this higher vaccine dose. The antiserum formed strong precipitin lines to the A and D antigens, and in IE it formed a weak line to the c antigen, indicating contamination of the A precipitin arcs with the other antigens. In the ID test the antiserum

formed lines of identity with the A lines formed by positive human sera (Fig. 5). Antibodies to the c antigen could be removed from the lot 4 serum by absorption (see below).

The lot 4 antiserum was absorbed with *B. dermatitidis* yeast-phase cell walls and cytoplasmic particles and with *H. capsulatum* yeast-phase cells. The results obtained in CF and ID tests before and after absorption are presented in Tables 2 and 3. After being absorbed with cell walls of the yeast phase of *B. dermatitidis*, the A and c precipitin lines were no longer present in the ID test, but the D precipitin line remained. This latter absorption also substantially reduced the CF titer to the three *B. dermatitidis* antigens and to histoplasmin but did not affect the titer to *H. capsulatum* yeast-phase CF antigen. Absorbing the lot 4 serum with the cytoplasmic particles of *B. dermatitidis* removed the c precipitin band in the ID test and substantially reduced the CF titers to *B. dermatitidis* yeast-phase CF antigen and cytoplasmic particles and to histoplasmin. The latter absorption did not

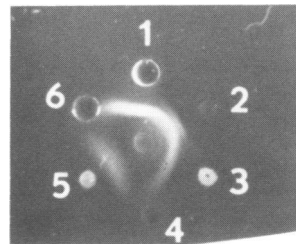


FIG. 4. ID test with the nonspecific lot 1C antiserum. Center well, lot 1C antiserum; well 1, *H. capsulatum* histoplasmin ID antigen; well 2, water-soluble antigen of *B. dermatitidis*; wells 3 and 5, ID antigen of *C. immitis*. Wells 4 and 6 were empty.

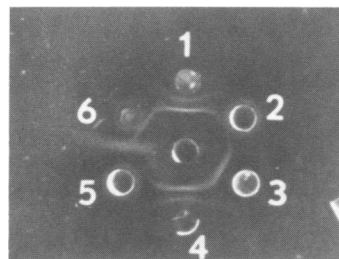


FIG. 5. Lot 4 anti-A-D serum used in identifying antibodies to the A antigen in human sera from patients with proven cases of blastomycosis. Center well, water-soluble antigen of *B. dermatitidis*; wells 1 and 4, lot 4 anti-A-D rabbit serum; wells 2, 3, 5, and 6, different human sera known to contain antibodies to the A antigen. The precipitin line between wells 5 and 6 is an unidentified serum to serum reaction. The precipitin lines closest to wells 1 and 4 are the D lines.

TABLE 2. Results of CF tests of absorbed and nonabsorbed *B. dermatitidis* lot 4 rabbit antiserum<sup>a</sup>

| Test antigen           | Dilution factor of CF titer with anti-serum nonabsorbed | Dilution factor of CF titer with the following absorbing antigens: |  |  |
|------------------------|---|--|--|--|
|                        |   | <i>B. dermatitidis</i> cell walls                                  | <i>B. dermatitidis</i> cytoplasmic particles | <i>H. capsulatum</i> yeast-phase cells |
| <i>B. dermatitidis</i> |   |  |  |  |
| Yeast-phase CF         | 512   | 64   | 64   | 128                                    |
| Cytoplasmic particles  | 64  | 0  | 0  | 0                                      |
| Water-soluble antigen  | 256   | 64   | 256  | 128                                    |
| <i>H. capsulatum</i>   |   |  |  |  |
| Yeast-phase CF         | 32  | 32   | 32   | 0                                      |
| Histoplasmin CF        | 16  | 0  | 0  | 0                                      |

<sup>a</sup> See Table 1, footnote a for preparation of antiserum.

TABLE 3. Results of ID tests of absorbed and nonabsorbed *B. dermatitidis* lot 4 rabbit antiserum<sup>a</sup>

| Test antigen            | Presence of antibodies <sup>b</sup> |                                   |  |  |
|-------------------------|-------------------------------------|-----------------------------------|--|--|
|                         | Nonabsorbed antiserum               | Absorbing antigens                |  |  |
|                         |                                     | <i>B. dermatitidis</i> cell walls | <i>B. dermatitidis</i> cytoplasmic particles | <i>H. capsulatum</i> yeast-phase cells |
| Water-soluble A antigen | +                                   | -                                 | +  | +                                      |
| Water-soluble D antigen | +                                   | +                                 | +  | +                                      |
| Water-soluble c antigen | w                                   | -                                 | -  | -                                      |

<sup>a</sup> See Table 1, footnote a for preparation of antiserum.

<sup>b</sup> +, Strong precipitin line; w, weak precipitin line; -, no line.

affect the CF titers to the *B. dermatitidis* water-soluble antigen or to *H. capsulatum* yeast-phase CF antigen. Serum absorbed with yeast-phase cells of *H. capsulatum* reacted only with the A and D antigens in the ID test and with *B. dermatitidis* yeast-phase antigen and water-soluble antigen in the CF test.

To date, no antibodies to the D antigen have been observed in sera from patients with blastomycosis, so that its significance is not yet known. This antigen does not react in the ID test with antisera to *C. immitis* or *H. capsulatum* and to this extent appears to be specific for *B. dermatitidis*. Therefore, the absorbed lot 4 serum can be used as a reference serum in the ID test and, if desired, as a positive control serum in the CF test. Like most rabbit sera, it is anti-complementary at the lower dilutions and there-

fore is not the most desirable positive control serum for the CF test.

**FA conjugate prepared from absorbed lot 4 antiserum.** The FA conjugate prepared from the immunoglobulin G fraction of lot 4 antiserum contained 6 mg of protein per ml and had a fluorescein-to-protein ratio of 12. The conjugate was tested against eight isolates of *B. dermatitidis*, five isolates of *H. capsulatum*, and 41 additional heterologous organisms (Table 3). All of the *B. dermatitidis* isolates except A295 stained at intensities of 3+ to 4+, except when an occasional cell stained at only a 2% intensity. Most of the individual cells of isolate A295 stained at 2+; however, a few cells in each microscopic field were not stained by the conjugate. A water-soluble antigen was prepared from this isolate and tested in the ID test. Qualitatively, this isolate contains less of the A antigen than did the other isolates tested, which would account for the weak staining intensity. The heterologous cultures were for all practical purposes not stained by the conjugate, although a few cells of *Sporothrix schenckii* and *Geotrichum candidum* were stained 1+ to 2+.

## DISCUSSION

The preparation of an antiserum in rabbits that is reactive with the A antigen of *B. dermatitidis* provides the first adequate supply of reference ID antiserum for the laboratory diagnosis of blastomycosis. This antiserum prepared

TABLE 4. FA staining of homologous and heterologous cultures with fluorescein isothiocyanate-labeled globulin from lot 4 antiserum

| Culture   | No. tested | Staining intensity                          |
|---|------------|---|
| <i>B. dermatitidis</i>  | 8          | 3+ to 4+                                    |
| <i>Candida albicans</i>   | 4          | Negative                                    |
| <i>Candida tropicalis</i>   | 2          | Negative                                    |
| <i>Candida stellatoidea</i>   | 2          | Negative                                    |
| <i>Candida krusei</i>   | 1          | Negative                                    |
| <i>Candida pseudotropicalis</i>                                       | 2          | Negative                                    |
| <i>Candida parapsilosis</i>   | 4          | Negative                                    |
| <i>Cryptococcus neoformans</i>  | 4          | Negative                                    |
| <i>Cryptococcus albidus</i>   | 2          | Negative                                    |
| <i>Cryptococcus laurentii</i>   | 2          | Negative                                    |
| <i>Cryptococcus leucoleus</i>   | 1          | Negative                                    |
| <i>Cryptococcus terreus</i>   | 2          | Negative                                    |
| <i>G. candidum</i>  | 2          | Negative (few spores 1+ to 2+)              |
| <i>H. capsulatum</i>  | 5          | Negative                                    |
| <i>Histoplasma capsulatum</i> var. <i>duboisii</i>                    | 2          | Negative                                    |
| Impression smear of tissue from mouse infected with <i>C. immitis</i> | 1          | Spherules negative; few endospores 1+ to 2+ |
| <i>Paracoccidioides brasiliensis</i>                                  | 3          | Negative                                    |
| <i>Rhodotorula rubra</i>  | 2          | Negative                                    |
| <i>S. schenckii</i>   | 2          | Negative; few cells 2+                      |
| <i>Torulopsis glabrata</i>  | 4          | Negative                                    |

with precipitin arcs from IE plates originally contained antibodies to several additional antigens of *B. dermatitidis*, a new antigen we are calling D, and one or more nonspecific antigens that appear to be the same as the c antigen described by Abernathy and Heiner (1). In the ID test antibodies to the latter antigens were not observed after absorption of the serum with the cytoplasmic particles of *B. dermatitidis*, and therefore this serum does not react with ID antigens of *H. capsulatum* or *C. immitis*. However, serum absorbed in this manner does react in the CF test with *H. capsulatum* CF antigen. When the anti-A serum was absorbed with yeast-phase cells of *H. capsulatum*, it did not react with *H. capsulatum* antigens in either the ID or CF test or with *C. immitis* antigen in the ID test. When an FA conjugate was prepared from the *H. capsulatum*-absorbed serum, it did not stain one or more isolates of 16 heterologous fungi, but did stain a few cells of *G. candidum*, *C. immitis* (endospores only), and *S. schenckii*. However, the majority of these cells did not stain. The reason for the difference in reactivity of the antiserum absorbed with the two antigens is not known. This could reflect an incomplete absorption of the nonspecific or c antibodies by the cytoplasmic particles, the increased sensitivity of the CF and FA tests compared with that of the ID test, or the presence of antibodies to a variety of antigens, some of which were not present on the absorbing antigens. Thus, although absorption of the serum with *H. capsulatum* yeast cells did not remove all cross-reacting antibodies, when used in the ID test with homologous ID antigen, only A and D precipitin lines were formed. Therefore, this can be used as a reference serum for identifying antibodies to the A antigen in sera from suspected cases of blastomycosis. The diagnostic significance of the D antigen is not known.

Recently, Kaufman and Standard (12) used this anti-A serum in the ID test for identifying the A antigen in exoantigens of *B. dermatitidis*. They recommend this procedure for the rapid identification of these cultures. Also, the FA conjugate prepared from this serum has been used routinely by the Mycology Division, Center for Disease Control, for presumptive identification of *B. dermatitidis* yeast-phase cells. They have found that this conjugate gives brighter staining than previous conjugates prepared with intact cells (9).

Antisera prepared against cell walls, cytoplasm, and cytoplasmic particles provided information on the location of the A and c antigens in yeast-phase cells. Antisera raised against the

cell walls formed strong precipitin lines against the A and D antigens and weak precipitin lines against the nonspecific c antigen. No attempt was made to obtain purified cell walls, because all we were trying to do in this part of the study was develop a strong anti-A serum to be used in IE. The results suggest that the A antigen is located in the cell walls. This conclusion is further substantiated by the fact that the FA conjugate prepared from the anti-A-D serum stained intact yeast-phase cells of eight isolates of *B. dermatitidis*. Antisera prepared against the cell walls, cytoplasm, and cytoplasmic particles formed precipitin lines against the c antigen. Thus, the c antigen is apparently located in the cell walls and the internal portion of the cells. The results do not clearly define the location of the D antigen. Although antibodies to this antigen were present in sera from rabbits immunized with cell walls, the latter was not a purified preparation and thus could have contained the D antigen as a contaminant. Also, cell walls did not absorb D antibodies from an anti-A-D serum, nor was the D antigen present in a water-soluble antigen of cell walls. Furthermore, the anti-A-D FA conjugate gave weak to negative staining of individual cells of *B. dermatitidis* isolate A295, which contains more c and D antigens than A antigen. The data suggest that the D antigen is not a surface antigen.

Cox and Larsh (3) tested the cytoplasm and an alkali-soluble-water-soluble extract of purified cell walls of yeast-phase *B. dermatitidis* for skin test antigens. The cytoplasmic antigen reacted similarly with *H. capsulatum*- and *B. dermatitidis*-sensitized guinea pigs. The alkali-soluble-water-soluble antigen was more specific, giving positive skin test results in 24 h with 69 and 8% of the *B. dermatitidis*- and *H. capsulatum*-sensitized guinea pigs, respectively. Lancaster and Sprouse (13) extended these studies by fractionating the alkali-soluble-water-soluble antigen with preparative polyacrylamide gel electrophoresis. They obtained four fractions, one of which produced a positive skin test only in *B. dermatitidis*-sensitized guinea pigs. Although apparently no attempt was made to identify these antigens serologically, our results suggest that the cross-reactivity of the cytoplasmic skin test antigen may be due primarily to the c antigen and that the more specific alkali-soluble-water-soluble antigen contains the A or other specific antigens. If our hypothesis is correct, the anti-A and anti-D sera will be useful tools for identifying and following the purification of a specific skin test antigen for blastomycosis.

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