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Isolation and Identification of an Exoantigen Specific for *Coccidioides immitis*

REBECCA A. COX* AND LORENE A. BRITT

Department of Research Immunology, San Antonio State Chest Hospital, San Antonio, Texas 78223

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Results of previous studies have established that mycelial-phase cells of *Coccidioides immitis* produce a heat-stable (HS) exoantigen that is specific for this fungus. In the present study, the HS exoantigen was isolated from a heterogeneous culture filtrate of *C. immitis* mycelia by using a combination of physicochemical procedures. Affinity chromatography of the culture filtrate on concanavalin A yielded two fractions: an effluent fraction that did not bind to the lectin and an eluate fraction that eluted in α-2-methylmannoside. Antigenic analyses by two-dimensional immunoelectrophoresis established that of the seven antigens detected in the unfractionated culture filtrate preparation, only two were present in the column effluent, and of these, only one was stable to heating at 56°C for 30 min. Reactivity in the immunodiffusion assay for the HS exoantigen was demonstrable with the column effluent fraction and not the column eluate. The detection of only one precipitinogen in two-dimensional immunoelectrophoresis of the heat-treated concanavalin A effluent fraction, coupled with the reactivity of this fraction in the immunodiffusion assay for the HS antigen, provides strong, if not definitive, evidence that this antigen is the HS exoantigen. Purification of the HS antigen and the production of monospecific antiserum will provide the necessary reagents for the development of a sensitive and specific immunoassay for detecting the HS antigen in *C. immitis* cultures.

Coccidioidomycosis is a mycotic disease that presents a diverse clinical spectrum ranging from asymptomatic infection to a chronic or progressive pulmonary or disseminated disease. Demonstration of skin test and serologic conversion to coccidioidal antigens are useful aids in establishing the diagnosis of coccidioidomycosis, but a definitive diagnosis is contingent on isolation of the etiologic agent *Coccidioides immitis* and identification of the morphologic conversion of the mycelial phase to the parasitic spherule-endospore phase. Problems are encountered, however, in that 5 to 25% of *C. immitis* isolates exhibit atypical cultural or morphologic characteristics or both (10, 12). Further complications arise from the morphologic similarity of certain saprophytic fungi, notably members of the family *Gymnoascaceae* which, like *C. immitis*, produce thallic arthroconidia (4, 16).

Identification of *C. immitis* cultures has been greatly facilitated by the studies of Kaufman and Standard (12, 13, 17). These investigators established that mycelia of *C. immitis* produce a heat-stable (HS) exoantigen that is specific for this fungus. Detection of the HS exoantigen is accomplished by immunodiffusion (ID) of the mycelial-phase culture filtrate against a reference coccidioidin (CDN)-anti-CDN system. Although the reference CDN and anti-CDN used in the ID assay for the HS antigen (IDHS assay) contain multiple reactants, the two reagents are titrated against each other to yield a single precipitin band that corresponds to a reaction between the HS antigen and the homologous antibody.

In a large series of studies, investigators (2, 3, 11–13, 17) established that the HS exoantigen is demonstrable in 100% of culture filtrates of *C. immitis* and in none of the extracts derived from heterologous fungi, including such pathogens as *Histoplasma capsulatum* and *Blastomyces dermatitidis* and several arthroconidia-producing saprophytes such as *Auxarthron* spp., *Arachiotus* spp., and *Malbranchea* spp. The utility of detecting the HS exoantigen in identifying *C. immitis* cultures prompted investigations to isolate and purify this precipitinogen for use as an immunologic reagent. In this report we summarize the results of these studies.

MATERIALS AND METHODS

Antigens. Four CDN preparations were employed. A broth culture filtrate antigen, designated CDN-F, was used in the isolation of the HS exoantigen. This CDN-F preparation was obtained from young mycelia of strain Silveira (ATCC 28868) by the method of Huppert et al. (8). In brief, mycelia were grown in a 2% glucose–1% yeast extract broth for 3 days at 33°C in a gyratory shaker at 120 rpm. The cultures were killed with Formalin (0.1%) and then filtered through Whatman no. 1 filter paper to remove intact mycelial cells. The filtrate was clarified by sequential passage through membranes (pore sizes, 3.0 and 0.45 μm) and then concentrated by ultrafiltration on a PM-10 membrane (Amicon Corp., Lexington, Mass.). The retentate was lyophilized and stored at −20°C until use.

The other three CDN preparations were employed in two-dimensional (2D) immunoelectrophoresis (IEP) for antigenic analyses and comparisons of fractions derived from CDN-F. One of these was a concentrated filtrate antigen which has served as a reference CDN for use in the IDHS assay (14). This reference filtrate antigen was generously provided by Leo Kaufman (Centers for Disease Control, Atlanta, Ga.) and was designated CDN-(R)F to distinguish it from the CDN-F extract prepared by us. A CDN prepared as a toluene-induced lysate of strain Silveira (15) was obtained from Demosthenes Pappagianis (University of California at Davis). This lysate antigen was designated CDN-L. The fourth CDN used in this study was prepared as a culture filtrate extract combined with the toluene-induced lysate of

* Corresponding author.

mycelial-phase cells of 24 *C. immitis* strains. This preparation was designated CDN-F/L and is the same reference CDN antigen prepared by Huppert et al. (9) for antigenic analysis of *C. immitis* antigens by 2D-IEP.

Antisera. Antiserum to CDN-L was obtained by immunizing a goat intramascularly with antigen (10 mg) in complete Freund adjuvant. Booster injections of antigen (5 mg) were given in incomplete adjuvant at monthly or bimonthly intervals. The immunoglobulin fraction was isolated by precipitation of sera with an equal volume of saturated ammonium sulfate. Precipitated immunoglobulins were dialyzed against distilled water and then lyophilized.

Hyperimmune burro anti-CDN was the same antiserum prepared previously by Huppert et al. (9) for antigenic analysis of *C. immitis* by 2D-IEP. This antiserum was obtained by immunizing a burro with CDN-F/L over a course of 1 year.

Affinity chromatography. Affinity chromatography was performed on columns (2.5 by 10 cm) containing concanavalin A (ConA) covalently linked to Sepharose 4B (Sigma Chemical Co., St. Louis, Mo.). Antigen (8 mg) was dissolved in 0.01 M Tris-buffered saline (pH 7.2) containing 10^{-4} M $CaCl_2$ and 10^{-4} M $MgCl_2$ and applied to columns preequilibrated with buffer. Nonbound components were eluted at a descending flow rate of 15 ml/h. After the A_{280} of the effluent returned to base line, bound components were eluted in buffer containing 0.2 M α-2-methylmannoside, a competitive inhibitor of ConA binding (5). The effluent and eluate fractions were dialyzed against distilled water and then lyophilized.

2D-IEP. The antigenic composition of CDN-F was assessed by 2D-IEP by previously published procedures (1, 18). Antigen (20 μl) was applied in a well (diameter, 5 mm) punched 2 cm from the side and bottom of a gel bond film (FMC Corp., Marine Colloids Div., Rockland, Maine) precoated with 1% agarose (type 1; Sigma). First-dimension electrophoresis was performed for 1 h at 10 V/cm. The agarose was removed 5 mm above the first dimension and replaced with 1% agarose containing immunoglobulin. Electrophoresis in the second dimension was performed at 3 V/cm for 17 h at 15°C. Nonprecipitated components were eluted from the gels with saline; and the gels were then washed in distilled water, dried, and stained with Coomassie brilliant blue R.

For line-IEP, reference antigen was subjected to electrophoresis in the first dimension. An agarose strip (width, 7 mm) containing CDN-F, derived fractions, or, for a control, buffer only was then poured parallel to the first-dimension gel. The intermediate gel was allowed to solidify, and agarose containing immunoglobulin was applied to the remainder of the second-dimensional gel area. Electrophoresis was then performed perpendicular to the first dimension at 3 V/cm for 17 h.

IDHS assay. Detection of the HS exoantigen was performed by the IDHS assay, as described by Standard and Kaufman (17). Reference CDN and anti-CDN were obtained from Nolan Laboratories (Atlanta, Ga.).

RESULTS

Fractionation of CDN-F. In initial experiments, CDN-F, CDN-L, and goat anti-CDN were evaluated by the IDHS assay to ensure that the two CDN antigens contained the HS exoantigen and that goat anti-CDN contained antibody to the HS exoantigen. All three reagents were reactive in the IDHS assay (data not shown), a result that established the validity of using CDN-F for the isolation of the HS exoantigen and

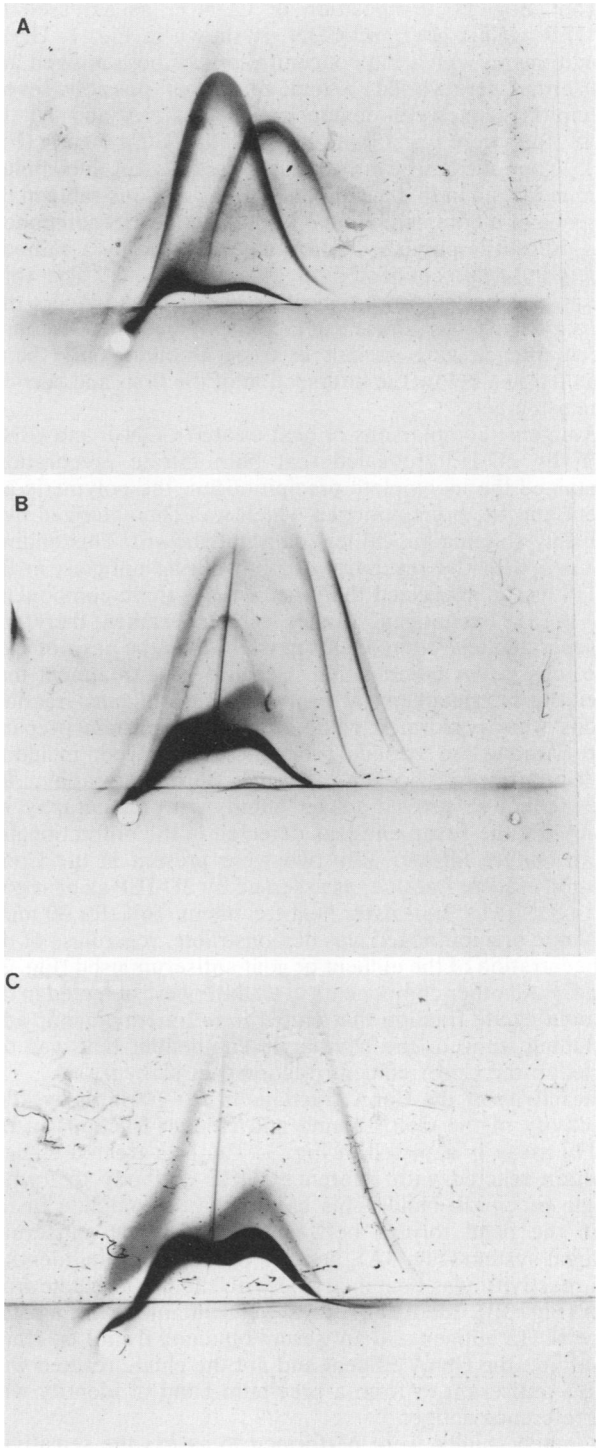

FIG. 1. Antigenic composition of CDN-F as assessed by 2D-IEP against goat anti-CDN. (A) Unheated CDN-F (100 μg) was assayed against an immunoglobulin concentration of 325 μg/cm² of gel; (B) unheated CDN-F (600 μg) against 810 μg of immunoglobulin per cm² of gel; (C) heat-treated CDN-F (600 μg) against 810 μg of immunoglobulin per cm² of gel. In the first dimension the anode is to the right; in the second dimension the anode is to the top.

CDN-L and homologous goat antiserum for monitoring the isolation of the HS antigen by 2D-IEP.

The antigenic composition of CDN-F, as assessed by 2D-IEP against goat anti-CDN, is shown in Fig. 1. Under conditions in which the concentrations of both antigen and antiserum were varied, a total of six, or possibly seven, precipitinogens were demonstrable (Fig. 1A and B). Of these, four were resistant to heating at 56°C for 30 min (Fig. 1C). One of these was characterized as an incomplete precipitinogen in that a cathodal leg was demonstrable in the absence of a complete anodal leg. A second HS component was present in polymeric form, as evidenced by a cathodal precipitin peak connected to an anodal peak. The third precipitinogen yielded a typical precipitin peak, except that the anodal leg was diffuse and incompletely stained. A fourth precipitinogen was present in trace amounts only, being detected just below the intersection of the first- and second-dimension gels.

Antigenic comparisons of heat-treated CDN-F and CDN-(R)F by 2D-IEP revealed that both filtrate preparations contained the incomplete precipitinogen, the polymeric antigen, and the precipitinogen which was characterized by a diffusely staining anodal leg (data not shown). This finding, coupled with the reactivity of both filtrate antigens in the IDHS assay, suggested that one of these three components was the HS exoantigen. Studies were undertaken, therefore, to separate these components in CDN-F on the basis of their size, charge, or composition. Because heat treatment may alter the physicochemical properties of antigens, fractionations were performed with the unheated filtrate preparation. Among the various procedures employed, including molecular-sieve and ion-exchange chromatography, the most effective proved to be affinity chromatography on ConA. Of the seven antigens detected in the unfractionated broth culture filtrate, only two were present in the ConA column effluent fraction, as assessed by 2D-IEP against goat anti-CDN (Fig. 2A). After heat treatment (56°C for 30 min), only one precipitinogen was demonstrable, regardless of the concentration of the effluent or goat antiserum used (Fig. 2B and C). All other components in CDN-F were detected in the column eluate fraction that eluted in α-2-methylmannoside, including some of the heat-labile component that was detected in the ConA effluent fraction (not shown).

Reactivity of the ConA fractions in the IDHS assay. The reactivity of the ConA-bound and effluent fractions in the IDHS assay is depicted in Fig. 3. The heat-treated effluent fraction reacted with reference IDHS antibody to form a single precipitin band. This band showed complete fusion with the band formed between the reference antiserum-antigen system (Fig. 3A), hence a reaction of total identity. No reactivity was demonstrable with the ConA eluate fraction (Fig. 3B). Identical results were obtained with a reference IDHS antigen and antiserum obtained from Leo Kaufman; i.e., the ConA effluent and not the eluate reacted with IDHS antiserum to form a precipitin band of identity with the reference antigen.

Limited studies were performed to assess the sensitivity and specificity of the HS ConA effluent antigen. Each of 16 successive lots of coccidioidin preparations yielded a precipitin band with reference IDHS antiserum that fused with the HS ConA effluent antigen. No reactivity was demonstrable in culture filtrates of six isolates of *H. capsulatum*, three isolates of *B. dermatitidis*, or one isolate of *Malbranchea dendritica*.

Antigenic homogeneity of the purified IDHS antigen. Detection of only one precipitinogen in the heat-treated ConA effluent fraction by 2D-IEP, together with the reactivity of this fraction in the IDHS assay, provided evidence that the heat-treated effluent fraction was antigenically homogeneous, containing only the HS antigen. To further assess the

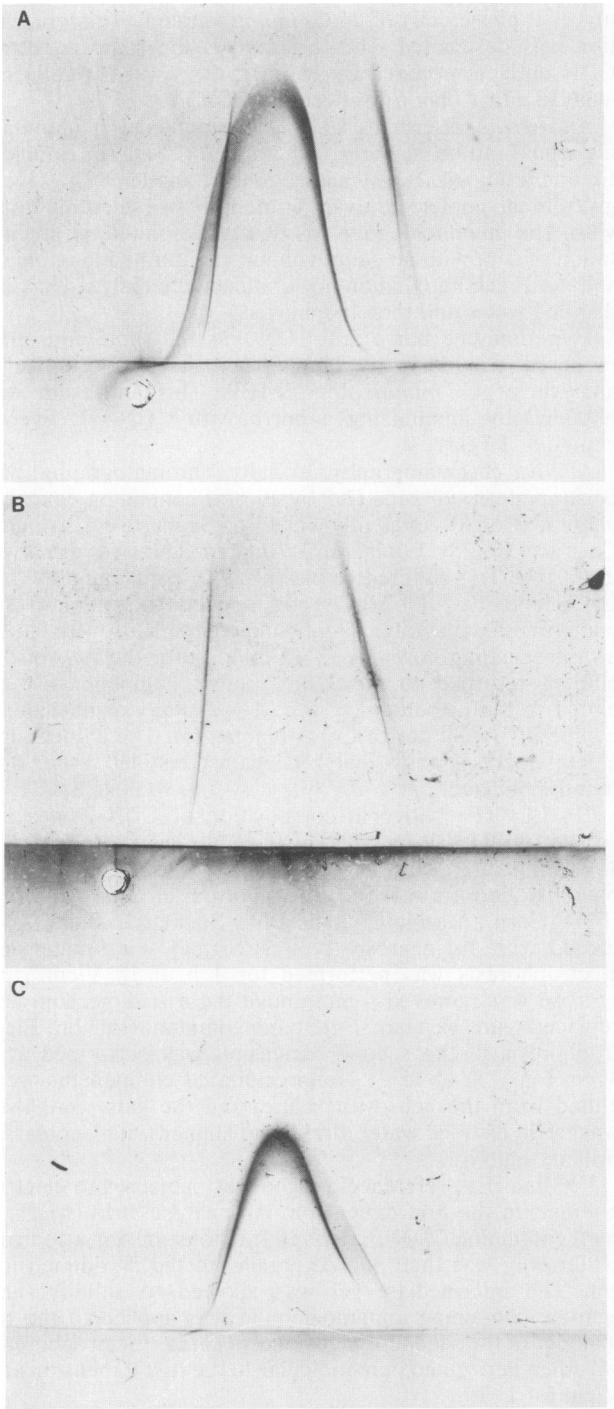

FIG. 2. Antigenic composition of the effluent fraction obtained by affinity chromatography of CDN-F on ConA as assessed by 2D-IEP against goat anti-CDN. (A) Unheated effluent (100 μg) was assayed against an immunoglobulin concentration of 405 μg of immunoglobulin per cm^2 of gel; (B) heat-treated effluent (100 μg) against 405 μg of immunoglobulin per cm^2 of gel; and (C) heat-treated effluent (50 μg) against 810 μg of immunoglobulin per cm^2 of gel.

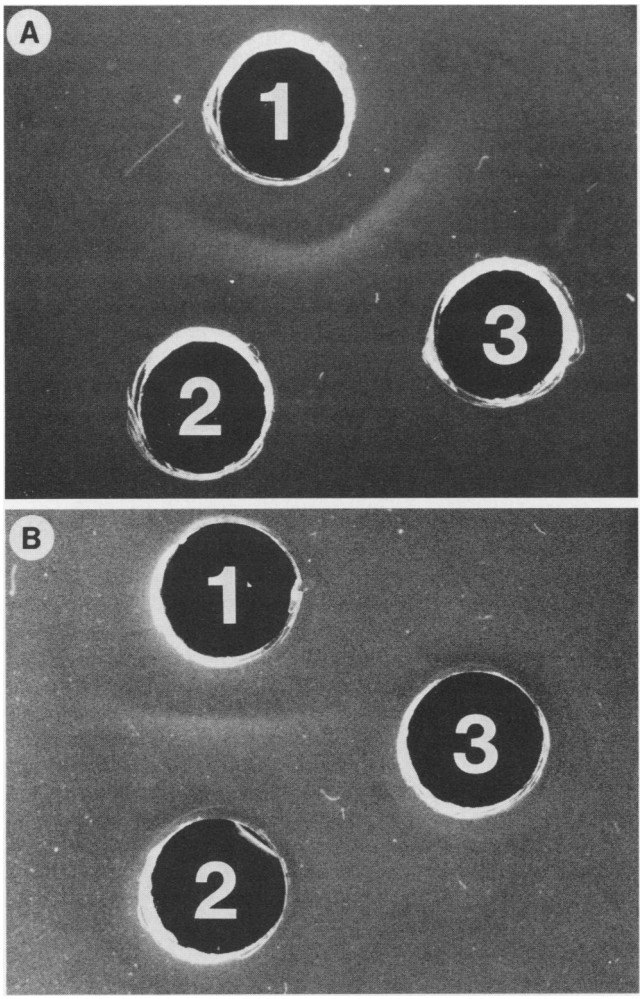

FIG. 3. Reactivity of the ConA effluent and eluate fractions in the IDHS assay. (A and B) Wells 1 and 2 contain reference IDHS antiserum and antigen, respectively. Well 3 contains the heat-treated effluent (250 μg/ml) in panel A and the eluate fraction (250 μg/ml) in panel B.

antigenic homogeneity of the purified HS antigen, the heat-treated effluent was subjected to line-IEP, i.e., interposed in a gel between the first-dimension gel electrophoresis of CDN-L and a second-dimension gel containing goat or burro anti-CDN.

The results obtained in line-IEP of the heat-treated ConA effluent in an intermediate gel between CDN-L and goat anti-CDN are shown in Fig. 4. The advancing line formed by the ConA effluent antigen in the intermediate gel (Fig. 4B) fused with one of the precipitin peaks in CDN-L, a result that establishes antigenic identity. No other CDN-L precipitin peaks were affected. Identical results were obtained in line-IEP of the effluent antigen with CDN-L against burro anti-CDN (Fig. 5) and with line-IEP of the effluent antigen with CDN-(R)F against goat anti-CDN (data not shown).

In a previous report, Huppert et al. (9) identified 26 precipitinogens in CDN-F/L by 2D-IEP against burro anti-CDN. The antigens were designated 1 through 26 on the basis of their relative migration velocity. Attempts were made to determine which of the reference CDN components corresponded to the HS exoantigen isolated in the present study. Line-IEP of the heat-treated ConA effluent fraction in the reference burro anti-CDN-CDN system resulted in the fusion of the HS antigen with a single component in CDN-F/L (Fig. 6). On the basis of the migration velocity of the latter reference CDN component relative to that of the polymeric antigen (antigen 2), we suspect that the HS antigen corresponds to the CDN-F/L component that was designated antigen 11. Unfortunately, this precipitinogen and many of the other antigens originally described in the reference CDN antigen preparation were reduced in peak height or were no longer demonstrable with burro anti-CDN, a finding that may be attributed to the loss of potency of the reference antigen, antiserum, or both.

DISCUSSION

A major goal of mycologists is the purification of fungal antigens with defined biologic activity. In the present study, a filtrate antigen obtained from 3-day-old cultures of *C. immitis* mycelia was purified by affinity chromatography on ConA followed by heat treatment of the HS-containing

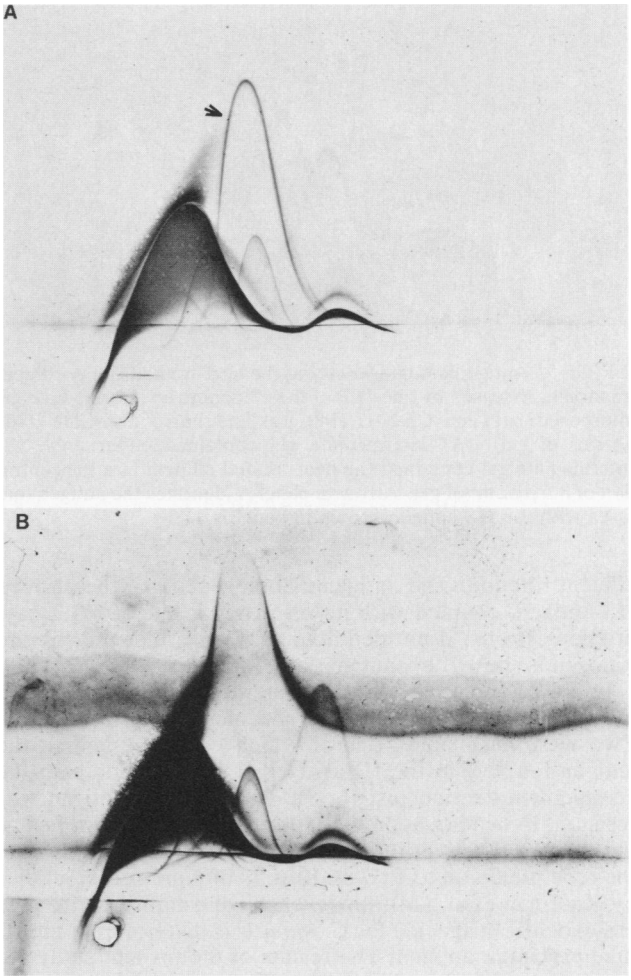

FIG. 4. Antigenic homogeneity of the heat-treated ConA effluent fraction as assessed by line-IEP of the effluent in an intermediate gel interposed beween CDN-L (300 μg) and goat anti-CDN (810 μg/cm^2 of gel). (A) Intermediate gel contained buffer only. (B) Intermediate gel contained the heat-treated effluent at a concentration of 93 μg/cm^2 of gel. Arrow in panel A identifies the antigen that fuses with the HS antigen shown in panel B.

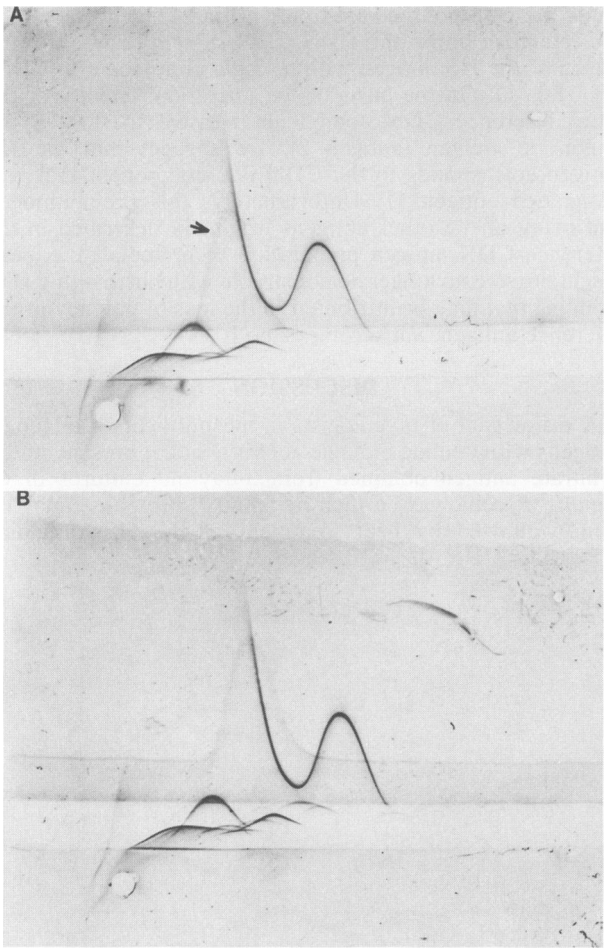

FIG. 5. Antigenic homogeneity of the heat-treated ConA effluent fraction as assessed by line-IEP of the effluent in an intermediate gel interposed between CDN-L (140 μg) and burro anti-CDN (405 μg/cm² of gel). (A) Intermediate gel contained buffer only. (B) Intermediate gel contained the heat-treated effluent at a concentration of 8.9 μg/cm² of gel. Arrow in panel A identifies the antigen that fuses with the HS antigen shown in panel B.

effluent fraction. The antigenic homogeneity of the purified HS antigen, coupled with its reactivity in the IDHS assay, provides strong, if not definitive, evidence that this precipitinogen is the HS exoantigen of *C. immitis*.

In the original development of the IDHS assay, three exoantigens were shown to be specific for *C. immitis* (17). Two were heat labile, one of which was identified as the antigen reactive in the ID assay that corresponds with the complement-fixation test (6, 8). The third exoantigen was resistant to heating at 56°C for 30 min and was identified as the antigen reactive in the ID assay that corresponds with the tube precipitin test (7, 8). Results of more recent studies by Kaufman et al. (14), however, have established that the HS exoantigen specific for *C. immitis* is distinct from the ID tube precipitin antigen. The results of the present study are consistent with this distinction. That is, reactivity in the IDHS assay was demonstrable with the ConA column effluent antigen, whereas reactivity in the ID tube precipitin assay was demonstrable with the eluate fraction (data not shown).

The reproducibility of the procedure used to isolate the HS exoantigen is contingent on the antigenic complexity of the CDN preparation used. In an effort to minimize antigenic heterogeneity, we used broth culture filtrates of young (3-day-old) mycelia. Although the HS antigen was demonstrable in culture filtrates of older mycelia and in toluene-induced lysates of mycelia, these preparations consistently contained more antigens, thereby reducing the efficacy of affinity chromatography.

Detection of only one precipitinogen in 2D-IEP of the heat-treated ConA effluent fraction provides evidence that this fraction is antigenically homogeneous. The possibility that other components, i.e., antigens that do not react as precipitinogens or nondialyzable media constituents, are also present in the ConA effluent fraction cannot be discounted. To circumvent this potential problem, studies have been undertaken to use the precipitin peak obtained in 2D-IEP of the HS antigen against goat anti-CDN as an immunogen. Although we are early in the course of immunization, the antiserum produced thus far appears to be specific for the HS antigen. Isolation of the HS antigen and production of monospecific antibody will provide the neces-

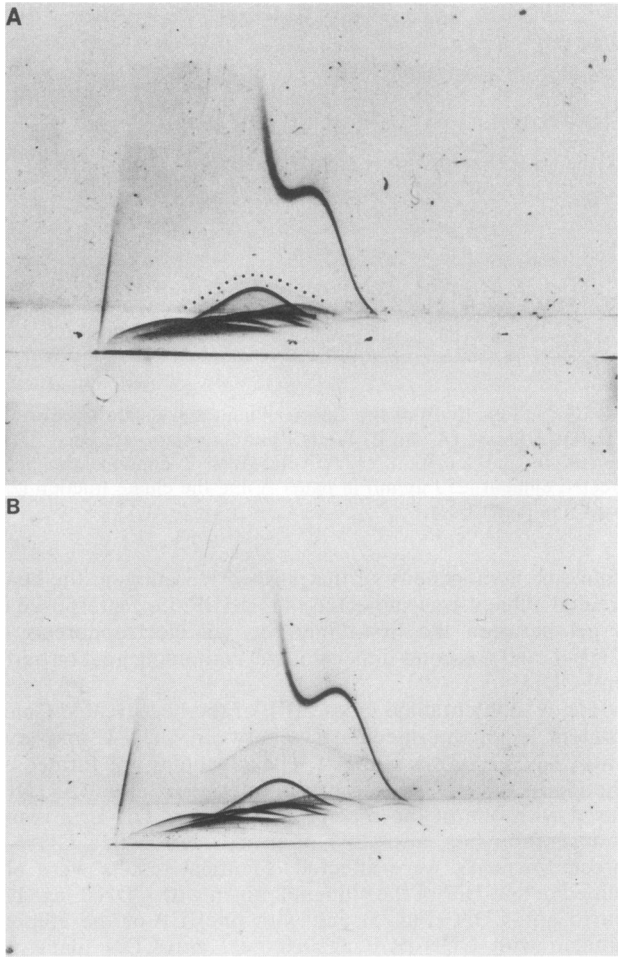

FIG. 6. Antigenic homogeneity of the heat-treated ConA effluent fraction as assessed by line-IEP of the effluent in an intermediate gel interposed between CDN-F/L (80 μg as protein) and burro anti-CDN (1:10). (A) Intermediate gel contained buffer only. (B) Intermediate gel contained the heat-treated effluent at a concentration of 8.6 μg/cm² of gel. Arrow in panel A identifies the antigen that fuses with the HS antigen shown in panel B.

sary reference reagents for the immunologic identification of *C. immitis* cultures.

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LITERATURE CITED

1. **Axelsen, N. H.** 1973. Intermediate gel in crossed and in fused rocket immunoelectrophoresis, p. 71–77. *In* N. H. Axelsen, J. Kroll, and B. Weeke (ed.), A manual of quantitative immunoelectrophoresis. Universitetsforlaget, Oslo.
2. **DiSalvo, A. F., A. S. Sekhon, G. A. Land, and W. H. Fleming.** 1980. Evaluation of the exoantigen test for identification of *Histoplasma* species and *Coccidioides immitis* cultures. J. Clin. Microbiol. **11**:238–241.
3. **DiSalvo, A. F., A. A. Terreni, and A. K. Wooten.** 1981. Use of the exoantigen test to identify *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Histoplasma capsulatum* in mixed cultures. J. Clin. Microbiol. **75**:825–826.
4. **Emmons, C. W.** 1967. Fungi which resemble *Coccidioides immitis*, p. 333–337. *In* L. Ajello (ed.), Proceedings of Second Coccidioidomycosis Symposium. University of Arizona Press, Tucson.
5. **Goldstein, I. J., C. E. Hollerman, and E. E. Smith.** 1965. Protein-carbohydrate interaction. II. Inhibition studies on the interaction of concanavalin A with polysaccharides. Biochemistry **4**:876–883.
6. **Huppert, M., and J. W. Bailey.** 1965. The use of immunodiffusion tests in coccidioidomycosis. I. The accuracy and reproducibility of the immunodiffusion test which correlates with complement fixation. Am. J. Clin. Pathol. **44**:364–368.
7. **Huppert, M., and J. W. Bailey.** 1965. The use of immunodiffusion tests in coccidioidomycosis. II. An immunodiffusion test as a substitute for the tube precipitin test. Am. J. Clin. Pathol. **44**:369–373.
8. **Huppert, M., J. W. Bailey, and P. Chitjian.** 1967. Immunodiffusion as a substitute for complement fixation and tube precipitin tests in coccidioidomycosis, p. 221–225. *In* L. Ajello (ed.), Proceedings of Second Coccidioidomycosis Symposium. University of Arizona Press, Tucson.
9. **Huppert, M., N. S. Spratt, K. R. Vukovich, S. H. Sun, and E. H. Rice.** 1978. Antigenic analysis of coccidioidin and spherulin determined by two-dimensional immunoelectrophoresis. Infect. Immun. **20**:541–551.
10. **Huppert, M., S. H. Sun, and J. W. Bailey.** 1967. Natural variability in *Coccidioides immitis*, p. 323–328. *In* L. Ajello (ed.), Proceedings of Second Coccidioidomycosis Symposium. University of Arizona Press, Tucson.
11. **Huppert, M., S. H. Sun, and E. H. Rice.** 1978. Specificity of exoantigens for identifying cultures of *Coccidioides immitis*. J. Clin. Microbiol. **8**:346–348.
12. **Kaufman, L., and P. Standard.** 1978. Immuno-identification of cultures of fungi pathogenic to man. Curr. Microbiol. **1**:135–140.
13. **Kaufman, L., and P. Standard.** 1978. Improved version of the exoantigen test for identification of *Coccidioides immitis* and *Histoplasma capsulatum* cultures. J. Clin. Microbiol. **8**:42–45.
14. **Kaufman, L., P. G. Standard, M. Huppert, and D. Pappagianis.** 1985. Comparison and diagnostic value of the coccidioidin heat-stable (HS and tube precipitin) antigens in immunodiffusion. J. Clin. Microbiol. **22**:515–518.
15. **Pappagianis, D., C. E. Smith, G. S. Kobayashi, and M. T. Saito.** 1961. Studies on antigens from young mycelia of *Coccidioides immitis*. J. Infect. Dis. **108**:35–44.
16. **Sigler, L., and J. W. Carmichael.** 1976. Taxonomy of *Malbranchea* and some other hyphomycetes with arthroconidia. Mycotaxonomy **4**:349–488.
17. **Standard, P. G., and L. Kaufman.** 1977. Immunological procedures for the rapid and specific identification of *Coccidioides immitis* cultures. J. Clin. Microbiol. **5**:149–153.
18. **Weeke, B.** 1973. Crossed immunoelectrophoresis, p. 47–56. *In* N. H. Axelsen, J. Kroll, and B. Weeke (eds.). A manual of quantitative immunoelectrophoresis. Universitetsforlaget, Oslo.