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Comparison of Immunoblot Analyses of Spherule-Endospore-Phase Extracellular Protein and Mycelial-Phase Antigen of *Coccidioides immitis*

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The extracellular proteins produced by *Coccidioides immitis* during growth of the spherule-endospore-phase and mycelial-phase antigen (coccidioidin) were studied by polyacrylamide gel electrophoresis followed by immunoblot analysis to detect specific serologic function. Filtrates obtained from 28- and 120-h growth of the spherule-endospore phase were compared with each other and with coccidioidin by using negative, immunoglobulin M (IgM) precipitin-positive, or complement fixation-positive pooled and single human sera followed by peroxidase-labeled anti-human IgA, IgE, IgG, or IgM (heavy chain specific) or peroxidase-labeled concanavalin A to detect the reaction. A total of 35 bands was seen in the stained gels. Different patterns were noted among the two spherule-endospore preparations and unheated and heated coccidioidin. At least 15 electrophoretically separate antigens were detected with positive serum ranging in approximate molecular weight (M_r) from 100,000 to 18,000. Most were clustered between 45 and 60 kilodaltons (kDa). Common bands were noted at 48 and 18 kDa. At least one band at 48 kDa was strongly reactive with complement fixation-positive serum demonstrated by reaction with anti-IgG and anti-IgE. In contrast, doublet bands in the 50- to 65-kDa area were highly reactive with IgM precipitin-positive serum detected by anti-IgM. IgM antibodies present in both positive sera reacted with a band at 46 kDa which was not reactive with IgG. Heating the antigens altered the reactivity of many of the antigens, including the 48-kDa band, but not the 46-kDa band.

Coccidioides immitis is a diphasic fungus that grows in hyphal-arthroconidial form in the soil of semiarid regions of the Western Hemisphere and under usual laboratory conditions. The infectious arthroconidia enter a susceptible host and become rounded and enlarge to form spherules, which undergo nuclear division and cleavage to form uninucleate endospores. These mature spherules rupture to liberate the endospores, which in turn enlarge to produce new spherules, thus completing the in vivo spherule-endospore (SE) life cycle (16). Under certain conditions the SE cycle can be maintained in vitro, wherein it shows a definite sequence of development with respect to cellular polymers and surface immunogens (11, 19, 20) and antigenic polymers released into the culture medium (35).

Serodiagnosis and monitoring of coccidioidomycosis entail detection of "precipitin" antibodies by tube precipitin, immunodiffusion (ID-TP), or latex particle agglutination in early coccidioidomycosis; and detection of complement-fixing antibody (CF)—also demonstrable by immunodiffusion (ID-CF)—later in the course of infection (14, 24, 31). The former antibody appears to be primarily immunoglobulin M (IgM); the latter appears to be primarily IgG (26, 28). The precise antigens of *C. immitis* for these two reactivities have not been defined, but differences are recognized, e.g., the antigen(s) reactive in tube precipitin or ID-TP (and delayed hypersensitivity) is heat stable, the antigen(s) reactive in CF or ID-CF is heat labile (14, 31). Coccidioidin, a mycelial-phase culture filtrate-autolysate, has customarily been used for serodiagnosis, but the SE phase also yields serologically active antigens.

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Furthermore, it was shown that these two antigenic activities appeared at different stages in the growth of the SE forms of *C. immitis* in vitro (35). The heat-stable ID-TP antigen was detectable in culture filtrates before spherules had undergone cleavage and before significant increases in soluble protein occurred. The heat labile ID-CF antigen was detected after conversion of endospores to immature spherules.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed some proteins associated with specific morphologic stages. The present study was undertaken to characterize electrophoretically separate antigens in two sequential SE phases and mycelial-phase (coccidioidin) culture filtrates. SDS-PAGE separation and nitrocellulose protein blots were utilized to identify culture filtrate components and their specific serologic reactivity.

MATERIALS AND METHODS

Mycelial-phase antigens. Two mycelial-phase culture filtrate antigens, or coccidioidins, were studied. The first, SF, was a mycelial culture filtrate of strain Silveira (ATCC 28868) (9). This antigen is routinely used in our laboratory to detect CF antibody by immunodiffusion with human and other sera. The second, F171, was a pooled filtrate of 22 strains of *C. immitis*. Heated F171 is routinely used to detect IgM precipitins by immunodiffusion tests of human and other sera. Both filtrates were analyzed before and after heating, which destroyed reactivity with CF antibody, but left intact ID-TP activity. These antigens were concentrated in an Amicon pressure cell with a UM 10 ultrafilter (molecular weight [M_r] cutoff, 10,000) to a protein concentration of 2 to 3 mg/ml.

Preparation of SE-phase antigens. The Silveira strain of *C. immitis* was grown in synchronous SE-phase culture by the

method of Levine et al. (19). Small endospores, obtained by differential centrifugation (11), were inoculated into 100 ml of chemically defined modified Converse medium to an absorbance at 440 nm of 0.02 (approximately 5×10^6 cells per ml) and incubated with shaking at 37°C. At 28 and 120 h after inoculation, culture samples were withdrawn and centrifuged at 400 × *g* for 15 min. The supernatants were filtered through 0.45-μm filters. Phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 1 mM to inhibit endogenous proteases. Samples of the 120-h filtrate were then concentrated in an Amicon pressure cell with a UM 10 ultrafilter to a protein concentration of 2 to 3 mg/ml.

To the 28-h culture filtrate, ammonium sulfate was added gradually to a final 90% saturation. This was stirred gently for 1 h at 4°C and then centrifuged at 31,500 × *g* for 45 min at 4°C in a Sorvall SS-34 rotor. The precipitates were dissolved in a minimal amount of 0.02 M potassium phosphate (pH 7.0) and dialyzed overnight at 4°C against the same buffer in EDTA-treated Spectrapor no. 3 tubing (M_r cutoff, 3,500). The retentate was further concentrated in a Savant Speed-Vac to a protein concentration of 2 to 3 mg/ml.

Both SE-phase filtrates possessed ID-TP and ID-CF antigens. After heat treatment, these filtrates retained ID-TP activity, but were no longer reactive with CF antibody in the immunodiffusion test.

Protein and carbohydrate determination. Protein determinations were carried out by the method of Lowry et al. (21) with bovine serum albumin (Sigma) as a standard. The anthrone method with mannose (Pfanstiehl Laboratories, Waukegan, Ill.) as a standard was used to detect nondialyzable hexoses (29).

Heat treatment. Samples (1 ml) of antigen at a protein concentration between 2 and 3 mg/ml were heated for 30 min a 60°C in a water bath.

Gel electrophoresis. SDS-PAGE was performed with the discontinuous buffer system of Laemmli (18). Samples were prepared for electrophoresis after reduction by the addition of at least an equal amount of the electrophoresis sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 2% SDS, 10% glycerol, 5% beta-mercaptoethanol, 0.002% bromphenol blue). Electrophoresis was carried out through both the 3.5% stacking gel and either 10 or 15% separating gels at 150 V for 4 h. The length of all gels was 9.25 cm. Gels were stained by either the silver stain as described by Merril (23), the periodic acid-Schiff stain (8), or Coomassie blue. Myosin (200 kilodaltons) [kDa], betagalactosidase (116 kDa), phosphorylase B (93 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa) (Bio-Rad Laboratories, Richmond, Calif.) were used as relative mobility standards.

Protein blot analysis. Protein blot analysis was performed by the method of Towbin et al. (33). Selected antigens were concentrated to 2 mg of protein per ml and electrophoresed as previously described. When electrophoresis was complete, the separated proteins were electrophoretically transferred to nitrocellulose paper (Schleicher & Schuell Co., Keene, N.H.; BA 85, 0.45 μm) at 200 mA for 18 h at 4°C. The nitrocellulose was either reacted immunochemically or stained with 0.1% amido black in 45% methanol and then destained with 10% methanol–7.5% acetic acid.

Immunochemical staining was performed by first blocking unbound sites on the nitrocellulose with 3% bovine serum albumin in TEN buffer (0.05 M Tris hydrochloride [pH 7.4], 0.001 M EDTA, 0.15 M sodium chloride) for 30 min at 4°C. The nitrocellulose was then cut into strips and incubated with the relevant human sera diluted in the range 1:25 to 1:100 in TEN buffer containing 0.1% bovine serum albumin. This incubation period was 2 h with constant agitation at room temperature. The blots were washed three times for 10 min each in TEN buffer. They were then incubated at room temperature for 2 h with constant agitation in horseradish peroxidase-labeled goat anti-human IgA, IgE, IgG, or IgM (heavy chain specific; Cooper Biomedical, Cochranville, Pa.) diluted from 1:250 to 1:1,000 in TEN buffer. The blots were again washed three times as before and developed with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.03% hydrogen peroxide in 0.1 M Tris hydrochloride (pH 7.4) for a maximum of 30 min. Separate blots were incubated with horseradish peroxidase-labeled concanavalin A (Sigma), which binds to glucose and mannose residues. The lectin concentration was 25 μg/ml. These blots were developed as described above.

Classification of human sera. Pooled positive human serum was used in protein blot analysis. These pools are routinely used as control sera in immunodiffusion testing for coccidioidal antibodies. The ID-TP serum was negative for CF antibodies, and vice versa, as measured by the immunodiffusion reaction. Pooled normal serum was also obtained from those submitted for serologic testing. This pool consisted of 50 sera negative for coccidioidal antibodies as determined after approximately eightfold concentration and testing by immunodiffusion.

Selected antigens were also reacted with 22 individual positive sera from patients with coccidioidomycosis and from patients with histoplasmosis (courtesy of N. L. Goodman, Department of Pathology, University of Kentucky Medical Center, Lexington, Ky.). The histoplasmal antisera showed no reactivity with coccidioidal antigens by immunodiffusion. In these experiments, all blots of each antigen were prepared from a single gel. The sera were diluted 1:50 and anti-IgM or -IgG was diluted 1:750.

RESULTS

Chemical composition of mycelial filtrates. The protein and carbohydrate contents of the stock mycelial filtrates were determined after dialysis of the stock. The antigen SF had 50 μg of protein per ml and 3.5 mg of carbohydrate per ml (ratio, 1:70). Heating did not alter these values. F171 contained 400 μg of protein per ml and 10 mg of carbohydrate per ml (ratio, 1:25). Heating did not change the protein concentration, but increased the concentration of measurable carbohydrate to 12 mg/ml.

The 28-h SE filtrate contained 30 μg of protein per ml and 115 μg of carbohydrate per ml (ratio, 1:4). The 120-h SE filtrate had 125 μg of protein per ml and 130 μg of carbohydrate per ml (ratio, 1:1). Heating did not alter either of these values.

Silver-stained SDS-PAGE comparisons of mycelial filtrates and SE-phase filtrates. Figure 1 illustrates the silver-stained electrophoretic pattern obtained under reducing conditions of the mycelial filtrates compared with the two SE-phase filtrates. The acrylamide concentration was 15%. Different patterns of bands were noted in the two SE preparations and in heated and unheated coccidioidin. Electrophoresis of F171, the 22-strain filtrate, revealed at least two major bands, identified by their approximate M_r of 48,000 and 18,000. Heating this antigen altered it by reducing the intensity of the 48-kDa band. At least three additional lower-M_r bands (42,000, 40,000, and 33,000) were produced by heating. The 18-kDa band was not changed.

Electrophoresis of the other mycelial antigen, SF, also revealed bands at 48 and 18 kDa. An additional band was present at 38 kDa. There was diffuse staining present at less than 14 kDa in both heated and unheated preparations. Heating SF completely destroyed the 48- and 38-kDa bands, but produced no new bands.

In comparison, electrophoresis of the 120-h SE filtrate showed at least 17 bands, including intensely staining bands present at 48 to 50 (triplet), 33, 31, 20, and 18 kDa as described previously (35). The bottom band of the triplet corresponded to the 48-kDa band described above for the mycelial antigens. As described previously (35), the 28-h SE filtrate presented a different electrophoretic appearance from the other antigens. At least 20 bands were present, including a strongly staining 18-kDa band. A definite 48-kDa band was not seen, although bands in that range were present.

Comparison of protein blot analyses of heated and unheated mycelial antigens with SE-phase antigens. Protein blots of the mycelial antigens SF, F171, and heated F171 were compared with blots from the 28-h and 120-h SE phase. These were reacted with normal, ID-TP, or pooled CF-positive human sera followed by either anti-human IgA, IgE, IgG, or IgM. Different patterns of electrophoretically separate antigens were noted among the two SE-phase antigens and heated and unheated mycelial antigens.

Figure 2 presents the protein blot analysis of the mycelial filtrate SF. This antigen contained at least seven immunoreactive bands (including three doublets) recognized by both ID-TP and CF sera. Normal serum did not react with these bands. The 48-, 38-, and 18-kDa bands described above as being silver stainable were reactive under some but not all conditions. The 48-kDa band was seen by all positive sera with anti-IgG, but not anti-IgM or -IgA, and only by ID-TP serum with anti-IgE. This 48-kDa band was demonstrable strongly only with CF serum followed by anti-IgG. It was not reactive with concanavalin A. The 38-kDa band was demonstrable with all positive sera with anti-IgG and -IgM, but not with anti-IgA or -IgE. The 18-kDa band was detected by all sera with anti-IgG and with normal serum only with anti-IgE. These two bands did not bind concanavalin A.

At least seven additional bands found in SF, not previously seen in stained preparations, were immunoreactive. These included bands ranging from 46 to 65 kDa. A band at 46 kDa, closely related to the 48-kDa band, was recognized by all positive sera with all anti-immunoglobulins. Three doublet bands in the 50- to 65-kDa region were also reactive with all positive sera. These were most strongly demonstrable with ID-TP serum detected with anti-IgM. The top two doublet bands were bound by concanavalin A, indicating the probable presence of mannose- or glucose-containing antigen.

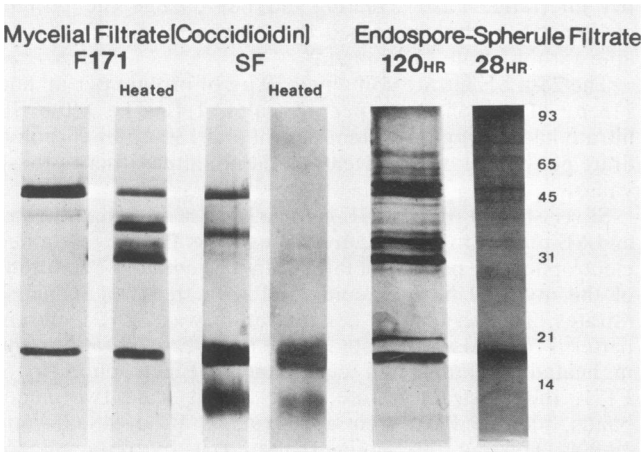

FIG. 1. Silver stain of SDS-PAGE of heated and unheated mycelial filtrates compared with the 28-h and 120-h SE-phase filtrates.

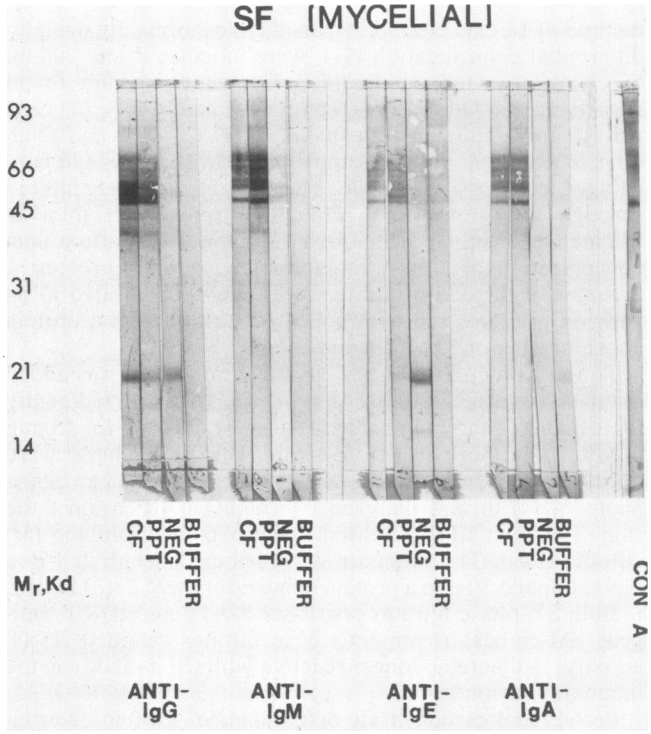

FIG. 2. Protein blot of the mycelial filtrate SF. CON A, Concanavalin A; PPT, precipitin serum; NEG, negative serum; complement-fixing serum.

The antigen F171 showed a similar, yet slightly different, pattern (Fig. 3). There were at least five immunoreactive bands in the range of 45 to 65 kDa, including the silver-stainable 48-kDa band. Both a 46-kDa band, not previously detectable, and the 48-kDa band were demonstrable intensely with CF serum followed by anti-IgG. The reactions of these two bands were also detected with ID-TP serum followed by anti-IgG or with either positive serum using anti-IgE as the detecting antibody. CF serum followed by anti-IgG and normal serum followed by anti-IgM were reactive with a 42-kDa band. The use of ID-TP serum followed by anti-IgM revealed a strong reaction with the 48-kDa band and at least three additional bands in the 50- to 65-kDa region. The entire 48-kDa band was also detected strongly by anti-IgA with either ID-TP or CF-reactive serum. The three additional bands in the 50- to 65-kDa region were likewise reactive with anti-IgA. In addition, a non-silver-staining 90-kDa band was seen. All positive sera followed by all anti-immunoglobulins were reactive with this band. Concanavalin A bound to all but two bands in the preparation.

Heating F171 altered the protein blots in several ways. CF serum followed by anti-IgG reacted with the three additional lower-M_r bands produced by heating this antigen. This included a strong reaction with the 40-kDa band. Both ID-TP and normal sera bound to the 42- and 40-kDa bands when either anti-IgM or -IgG was the detecting antibody. Concanavalin A bound to all bands in the blot of heated

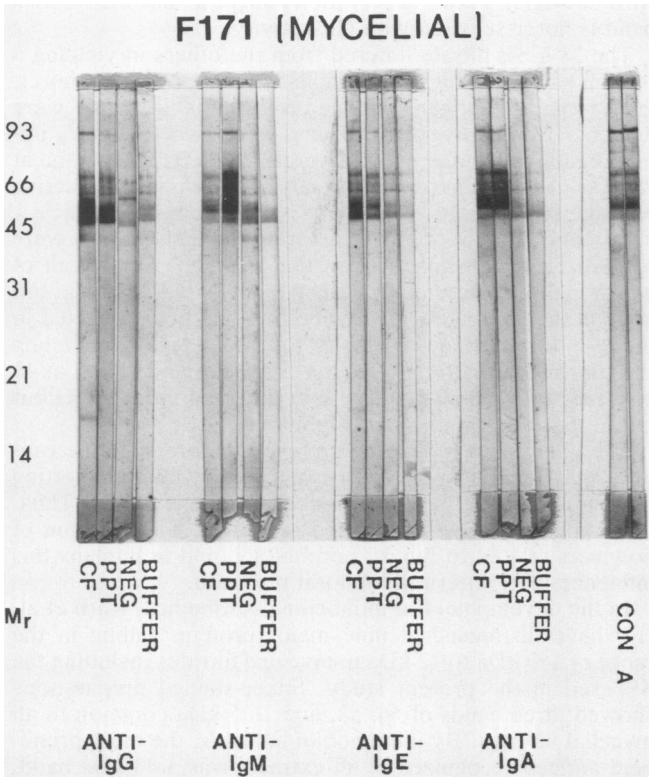

FIG. 3. Protein blot of the mycelial filtrate F171. Abbreviations are as in Fig. 2.

F171. Most importantly, when anti-IgM or -IgA was the detecting antibody, CF serum failed to bind to any of the bands of the heated F171, including those corresponding to bands previously detected in unheated coccidioidin. ID-TP serum followed by anti-IgM still recognized bands in the 50- to 65-kDa area, but this reaction was very slight compared with that observed with unheated antigen.

The protein blot analysis of the 28-h filtrate presented an electrophoretic appearance different from those of the above antigens (Fig. 4). At least 12 bands in the 100- to 46-kDa region were recognized by positive sera, including diffuse bands in the 45- to 50-kDa area. Heating the antigen eliminated the reactivity of many of the higher-M_r bands (greater than 65 kDa). The diffuse band at 45- to 50 kDa was not altered.

The protein blot analysis of the 120-h SE filtrate revealed at least 15 separate antigens from 95 to 28 kDa recognized by positive sera (data not shown). All of them had been previously detected by silver staining. The triplet bands, including the 48-kDa band, was recognized by anti-IgG and -IgE using either ID-TP or CF serum. The 48-kDa band was nonreactive with concanavalin A. Heating the antigen destroyed the immune reactivity of the triplet band, correlating with the results visualized in silver-stained gels.

The antigenic components of the 120-h SE-phase filtrate provided a heterogeneous pattern of immunoreactivity, with some reactions (e.g., the 48-kDa protein reactive with CF antibody revealed by anti-IgG) similar to those observed with other antigens, but some (e.g., the 50-kDa protein reactive with all positive sera detectable by any immunoglobulin) different from the reactions noted with mycelial antigens.

Protein blots with single-donor sera. To determine the specificity of the results obtained with pooled sera, protein blot analyses of heated and unheated antigens were performed with single-donor sera. These immunoblots in general corresponded to those seen previously in the pooled serum experiments. The 48-kDa band was prominent with all CF sera detected by anti-IgG; the immunoreactivity of this band was destroyed or reduced by prior heating. Two anti-*Histoplasma capsulatum* sera detected the band weakly with anti-IgG, but not with anti-IgM, in blots of the mycelial filtrate SF. In blots of the SE phase, the anti-*H. capsulatum* sera did not recognize any bands, regardless of the detection of antibody.

DISCUSSION

The filtrates derived from the SE and mycelial phases differed markedly in the relative amounts of polysaccharide and protein. The 28- and 120-h SE filtrates contained protein/carbohydrate ratios, respectively, of 1:4 and 1:1 (wt/wt). By contrast, the mycelial antigen preparation SF had a protein/carbohydrate ratio of 1:70, which was not altered by heating at 60°C. Mycelial preparation F171 had a protein/carbohydrate ratio of 1:25 in the unheated form; this was altered to 1:30 by heating at 60°C (measurable carbohydrate increased from 10 to 12 mg/ml). These values resemble the average relative amounts of polysaccharide and protein determined in prior chemical studies on coccidioidin (12, 25).

The increase in carbohydrate in F171 after heating may reflect the presence of neutral sugars or glucosamine held in strain-dependent cross-linkages with peptides which become anthrone reactive only after some type of protein denaturation. Heat denaturation, usually associated with a loss of biological activity, generally breaks the ionic and covalent bonds that give proteins their secondary and tertiary struc-

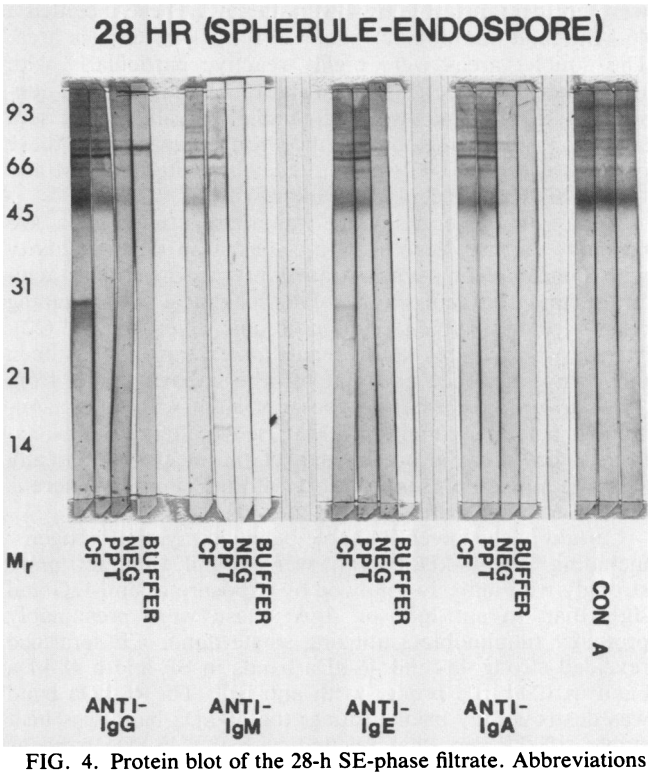

FIG. 4. Protein blot of the 28-h SE-phase filtrate. Abbreviations are as in Fig. 2.

ture and hence their conformation (32). The possible presence of cross-linked form of chitin-peptide in *C. immitis* cell walls was suggested by Wheat et al. (34) from their finding that some glucosamine was lost during digestion with pronase.

Electrophoresis and silver staining demonstrated 20 bands in the 28-h SE filtrate and 17 bands in the 120-h SE filtrate; mycelial F171 and SF had 5 and 4 bands, respectively. Although these differences were noted between SE and mycelial preparations, common bands were noted in the 48- and 18-kDa areas in the two SE preparations and in heated and unheated coccidioidin. The 48-kDa band of F171 became less intense than the corresponding band in unheated antigen; the 48-kDa band of SF disappeared after heating.

Protein blot analysis of unheated mycelial antigens revealed many immunoreactive bands that corresponded to silver-stainable bands. Significantly, the major 48-kDa band was recognized in F171 by all positive sera whether they contained ID-TP, CF, or both reactive antibody types with anti-IgG and -IgA. In SF the 48-kDa band, although demonstrable by all positive sera followed by anti-IgG, was strongly reactive only with CF-positive sera followed by anti-IgG. The 48-kDa band also yielded a slight reaction when normal serum or buffer was used as a result of nonspecific binding of the anti-immunoglobulin.

Protein blot analysis also showed that the 48-kDa band was not detectable after the mycelial filtrate SF had been heated. Heating followed by reduction of F171 resulted in a decrease of silver-stainable 46- and 48-kDa bands, yet both of these retained some reactivity on immunoblots with CF serum followed by anti-IgG. Two silver-stainable bands of lower molecular weight were also still demonstrable after heating. However, no bands were detectable with the same CF serum when anti-IgM or -IgA was used as a probe.

However, there were also major immunoreactive bands in both mycelial antigens detected by the blot technique that were not demonstrable by silver staining. These included a 46-kDa band and doublet bands in the 50- to 65-kDa area. The doublet areas were highly reactive particularly with ID-TP serum detected by anti-IgM. Although these non-silver-stainable bands were detectable with all positive sera and all anti-immunoglobulins, the strongest reaction of these bands was with ID-TP serum detectable with anti-IgM and -IgA. This was particularly evident with F171.

These non-silver-stainable immunoreactive bands are probably carbohydrate in nature, based on their reactivity with concanavalin A and stainability with periodic acid-Schiff stain (data not shown). Photochemical silver staining results from selective reduction of ionic silver to metal (22). Many carbohydrates fail to react; for example, it has been our own experience and that of others that mannan from *Saccharomyces cerevisiae* does not stain with silver (Au-Young, personal communication; 1). An alternate explanation is that there is not enough of the electrophoretically separate antigen present to react with the stain, but there is enough to react with antibody.

Certain of the silver-stainable bands in mycelial antigens, including the 48-kDa band, were found to react more strongly with antisera followed by exposure to anti-IgG and -IgE than to anti-IgM or -IgA; these were presumably proteins. Immunoblots utilizing single donor CF sera also revealed strong 48- and 46-kDa bands in SF and a 48-kDa band in 120-h SE probed with anti-IgG. The 48-kDa band was destroyed by prior heating; the 46-kDa band was heat stable. ID-TP sera that reacted with the 46-kDa band as detected by anti-IgM did not reveal that band when probed with anti-IgG. These observations suggest that the 46-kDa band is not associated with CF activity.

The 28-h SE filtrate differed from the others in yielding a preponderance of higher-M_r bands evident as electrophoretically separate antigens between 65 and 100 kDa that were detected by positive sera. There were two lower-M_r immunoreactive regions, a diffuse area at 48 kDa and a band at 28 kDa. All immunoreactive bands and areas were reactive with the silver stain.

In contrast, 15 possible different antigen bands were recognized by positive sera in the 120-h SE filtrate, all of which had been previously detectable by silver staining, but none in the 65- to 100-kDa region that had been detected in the 28-h filtrate. Bands in the 45- to 50-kDa region (including the triplet) were strongly reactive. A heat-labile band at 48 kDa reacted with all positive sera detected by anti-IgG but not in those detected by anti-IgM.

The differences in polymers present before spherule rupture and after endospore release detected by immunoblotting correlate with the patterns of silver-stainable bands. Thus, there appears to be a regulated sequence of production of polymers related to fungal morphology, and in humans this influences the pattern of humoral response.

In the development of monoclonal antibodies, Karu et al. (17) have distinguished nine major proteins falling in the range of 150 kDa to 12 kDa in mycelial filtrates including the SF used in the present study. Silver-stained preparations showed three bands of 66, 53, and 16.5 kDa common to all mycelial filtrates. By immunoblot analysis, the most prominent antigen recognized in all extracts was a 43-kDa band. Their results suggested that some antigens that were strongly reactive with human sera did not induce antibody production in mice.

Calhoun et al. have analyzed three coccidioidal extracts by immunoblotting techniques with sera from patients infected with *C. immitis* (D. L. Calhoun, E. O. Osir, J. N. Galgiani, and J. H. Law, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 774, 1985); 9 of 10 patients had antigens of 100, 60, and 48 kDa. Four of five CF-reactive sera studied had IgG binding to a 45- to 48-kDa antigen, similar to our results.

Our Western blots appeared to have distinguished two major antigenic subunits: one or more of greater than 50 kDa, not stainable with silver, but stainable with periodic acid-Schiff stain and reactive with concanavalin A that were particularly reactive with ID-TP sera as detected by anti-IgM and -IgA; and others, particularly the 48-kDa protein subunit that reacted strongly with CF sera as detected by anti-IgG and -IgE.

Denaturation of the antigen by SDS-PAGE procedures left intact the ability of this 48-kDa subunit to bind antibody. Prior heat treatment (60°C, 30 min) destroyed or reduced this band. Both processes destroy the native conformation of proteins, but by different mechanisms. The presence of the 48-kDa band in unheated antigen on reducing SDS-PAGE coupled with the absence of the 48-kDa band after the lengthy heat treatment provides evidence for the designation of that reduced protein subunit as heat-labile and thus a probable correlation with heat-labile CF activity. Unequivocal correlation of a band with a known function is difficult, particularly when the resolution of such a band depends on its denaturation by SDS-PAGE (10). However, the correlation has gained support from our recent column chromatographic separation of fractions of mycelial filtrate SF yielding a protein (lacking mannose or glucose) with an average molecular weight of 120,000. This protein, upon reduction,

produces the 48-kDa protein detected in SDS-PAGE. This forerunner 120-kDa protein contains the ID-CF reactivity.

The multiple bands observed on electrophoresis and protein blots with mycelial and SE filtrates confirm the multiple antigenic activities reported by Huppert et al. (15), based on two-dimensional immunoelectrophoresis against serum from a burro hypersensitized with mycelial antigen. Twenty-six separate antigens were detected in coccidioidin, 10 of which were also present in spherulin. The latter possessed two antigenic species not present in the mycelial preparation (or present in concentrations too low to be detected by two-dimensional immunoelectrophoresis). Cox et al. (7) have recently added to the information indicating that antigen 2 has reactivity conforming to the ID-TP antigen, indicating, as did Collins et al. (3), that this antigen is associated with the cell wall. Cole et al. (4) demonstrated the presence of antigen 2 in the walls of arthroconidia and spherules.

The detection of antigen reactive with IgE, usually present only in nanogram amounts in sera, correlates well with the findings of Cox and Arnold (5), who reported that a significant proportion of patients with coccidioidomycosis showed elevated IgE levels. Production was not limited to anti-*C. immitis* IgE (6).

Several bands present in these blots were reactive with *H. capsulatum*-positive sera, including a very weak, irregular reaction with the 48-kDa band. This irregular serological cross-reactivity between *H. capsulatum* and *C. immitis* has been well documented (2, 30). The chemical nature of the coccidioidins was shown early to be similar to that of histoplasmin (27). In the two-dimensional electrophoretic system of Huppert et al. (13), *H. capsulatum* was shown to share 12 antigens with coccidioidin.

Based on the present study and other reports, we propose that polysaccharide antigen located in the cell wall is shed or secreted and elicits the IgM (TP, ID-TP) response usually observed in early coccidioidomycosis. On the basis of the temporal appearance of the CF activity and of the 48-kDa protein subunit (only in lysates or after spherule wall rupture), we propose that the latter (or a forerunner polymer) is derived from the cytoplasm. During replication of the SE phase in the chronically ill host, continuous production of the 48-kDa protein forerunner polymer would lead to a rise in CF titer, generally associated with extending coccidioidomycosis.

Thus, a variety of electrophoretically separate antigens have been identified in these studies to be reactive with positive patient sera. Further characterization of several of these serologically important antigens is in progress.

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