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Antigen Complex of *Coccidioides immitis* Which Elicits a Precipitin Antibody Response in Patients

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The occurrence in patients of elevated levels of immunoglobulin M (IgM) precipitin antibody to *Coccidioides immitis* antigens, which are commonly detected by the immunodiffusion-tube precipitin (TP) assay, is suggestive of primary nondisseminating coccidioidomycosis. We previously demonstrated that the concanavalin A-bound mycelial culture filtrate plus lysate preparation is a source of at least two TP antibody-reactive antigens (TP-Ags), which were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as 120- and 110-kDa fractions. Evidence is presented here that the crude filtrate plus lysate preparation contains additional lectin-bound, TP antibody-reactive fractions as well as a component which elicits a complement fixation antibody response in patients. The 120- and 110-kDa fractions were isolated from the antigen complex and further characterized in this paper. Both TP-Ags are glycoproteins and have been shown by immunoelectron microscopy to be colocalized within cytoplasmic vesicles and the wall of spherules. Deglycosylation of these TP-Ags by sodium periodate treatment resulted in a loss in patients of 82 to 95% of IgM adsorption to the antigens as detected by the enzyme-linked immunosorbent assay (ELISA). Comparison of their carbohydrate compositions revealed that mannose and glucose are the predominant monosaccharides of both TP-Ags but only the 120-kDa fraction contained 3-*O*-methylmannose, a sugar which appears to be unique to *C. immitis* among the systemic fungal pathogens. We previously showed that 3-*O*-methylmannose is at least partly responsible for the reactivity of IgM antibody with the 120-kDa TP-Ag. Good correlation was shown between results of immunodiffusion-TP assays and ELISAs of IgM response to both the 120- and 110-kDa fractions by using 70 serum samples from patients with proved coccidioidomycosis. However, only 2.8% (3 of 109) of the serum samples from patients with other mycoses and nonmycotic infections showed IgM adsorption to the 120-kDa TP-Ag as detected by the ELISA, while 21.1% (23 of 109) showed IgM adsorption to the 110-kDa TP-Ag. The 120-kDa TP-Ag is a potentially valuable serodiagnostic reagent for detection of specific IgM by ELISA in patients with primary coccidioidomycosis.

Serologic tests are valuable aids in the diagnosis of coccidioidomycosis (24), a respiratory disease of humans caused by the fungus *Coccidioides immitis*. The presence of elevated levels of immunoglobulin M (IgM) and IgG antibody to *C. immitis* antigen is recognized during the course of this disease (30, 31), and their detection is pivotal for the diagnosis of different clinical forms of coccidioidomycosis (24). The occurrence of IgM precipitin antibody to *C. immitis* antigen early in the infection process is characteristic of acute primary coccidioidomycosis but may also be detected during reactivation of the disease (1). In primary, nondisseminating coccidioidomycosis the appearance of anti-*C. immitis* IgM precipitin antibody is followed by a rising titer of anti-*C. immitis* IgG complement-fixing antibody. The titer of precipitin antibody commonly diminishes beginning at weeks 3 to 4 of illness, while the IgG titer continues to increase (24). Pappagianis and Zimmer (24) have emphasized that it is important to test for anti-*C. immitis* IgM antibody since this may be the only means of readily confirming the presence of an early acute coccidioidal infection, which would signal for timely antifungal drug therapy. Immunodiffusion (ID) assays are frequently used for detection of tube precipitin (TP) and complement fixation (CF) antibody to *C. immitis* antigens in patients (14–16). However, limitations in the successful clinical application of these and other methods of detection of serum antibodies in coccidioidomycosis are recognized (18, 25). The shortcomings are in part due to a lack of precise compositional data on the *C. immitis* antigens employed which makes standardization of the tests difficult to establish, as well as to serologic cross-reactivity with sera derived from patients with histoplasmosis, blastomycosis, and certain nonmycotic conditions (21).

Numerous preparations of *C. immitis* antigens have been used for assays which detect TP antibody in patients including the mycelial culture filtrate, filtrate plus toluene lysate of the isolated mycelia (F+L fraction), autolysate of the mycelial phase and parasitic cells (spherules plus endospores), and alkali-soluble, water-soluble extracts of isolated mycelial, conidial, and spherule wall fractions (10, 12, 17, 24, 37). In a recent study it was shown that concanavalin A (ConA) separation of the spherule culture filtrate permitted isolation of an affinity-bound fraction rich in TP antigen and with relatively few contaminating antigens (12a). We have reported that two electrophoretically distinct TP antigens can be initially separated from the F+L fraction of *C. immitis* by ConA chromatography (17). These two fractions were purified and identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as 120- and 110-kDa glycoproteins (6, 17). Resnick and coworkers (27) have reported that a 21-kDa serine proteinase isolated from the culture filtrate of the parasitic phase of *C. immitis* also demonstrates reactivity with precipitin antibody from sera of patients with coccidioidomycosis. It appears, therefore, that TP antigen preparations obtained from either the saprobic or parasitic phase of *C. immitis* are actually complexes of several reactive components. In this paper we present additional evidence that several macromolecules of *C. immitis* are

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capable of eliciting patient TP antibody response. Data are also presented which further characterize the physicochemical nature and immunologic reactivity of the purified 120- and 110-kDa components of the TP antigen complex.

MATERIALS AND METHODS

Cultivation. Arthroconidia of *C. immitis* C634, C735, and Silveira (ATCC 28868) were produced and harvested as previously described (9). Conidia of each strain were used separately to inoculate culture flasks containing glucose-yeast extract liquid medium (8). Abundant mycelia were produced after incubation of the flasks in a gyratory shaker (100 rpm) at 30°C for 5 days. Spherules of *C. immitis* (4) were grown in liquid Converse medium incubated at 39°C for 48 h as previously reported (8) and then harvested in preparation for immunoelectron-microscopic examinations as described below.

Preparation of crude antigenic fractions from the saprobic phase. The mycelial mat was separated from the culture supernatants of the three strains by filtration. The filtrates were pooled and dialyzed against distilled water (four changes over 48 h at 4°C) by using cellulose dialysis tubing with an estimated molecular mass cutoff of 6 to 8 kDa (Medical Industries Inc., Los Angeles, Calif.). The retained material (the mycelial culture filtrate fraction) was lyophilized and stored at −20°C. The combined mycelial mat from the three strains was washed with distilled water and then extracted with 3% (vol/vol) toluene (Fisher Scientific, Pittsburgh, Pa.) in distilled water for 3 days at 30°C as described by Pappagianis and coworkers (23). After centrifugation, the supernatant of the toluene lysate was dialyzed as described above and the retained material (the toluene lysate fraction) was lyophilized and stored at −20°C. The total lyophilized F+L fraction was finally solubilized in 0.02 M citric acid-Na_2HPO_4 buffer (pH 7.6) containing 0.5 M NaCl, 2.5×10^{-6}M $MnCl_2 \cdot 4H_2O$, 9.0×10^{-6} M $CaCl_2$, and 0.02% (wt/vol) NaN_3. The F+L mixture was the source of the TP antibody-reactive antigens (TP-Ags) examined in this study.

Fractionation of the mycelial F+L antigen complex. The lyophilized F+L mixture (10 mg), which was resolubilized in citric acid-Na_2HPO_4 buffer, was initially fractionated by ConA affinity chromatography as previously reported (17). The eluate fractions (ConA bound) showing the highest A_{280} were pooled, dialyzed against distilled water, and lyophilized as described elsewhere (17). This ConA-bound material was resolubilized in 10 mM Na_2HPO_4 (pH 7.0) containing 0.15 M NaCl at a concentration of 10 mg/ml and subjected to a second fractionation procedure. Aliquots (200 μl) of the resolubilized material were separated by high-pressure liquid chromatography (HPLC) by using an ion-exchange (DEAE) column (Protein Pak DEAE 5PW, 10-ml bed volume; Millipore Corp., Bedford, Mass.). Fractions were eluted with sample buffer plus 0.25 to 1.0 M NaCl with the following salt gradient (0.25 M, 0 to 10 min; 0.25 to 0.5 M, 10 to 20 min; 0.5 M, 20 to 35 min; 0.5 to 1.0 M, 35 to 60 min). Selected fractions with the same retention times from multiple sample separations were pooled, concentrated by centrifugation ($700 \times g$, 5 min, 4°C) with a Micropartition System MPS-1 (Amicon Corp., Danvers, Mass.) equipped with a YMT ultrafiltration membrane (10,000-molecular-mass cutoff; Amicon), and washed four times with phosphate-buffered saline (PBS; pH 7.6) containing 0.15 M NaCl. Each of these concentrated fractions was subsequently tested in ID assays for its ability to elicit a TP or CF antibody response in patients as described below. The composition of the ID-TP-positive fractions was examined by gel electrophoresis and ConA blotting as previously described (17). Briefly, samples were separated by SDS-PAGE conducted under reducing conditions (17) by using either a 7.5% or a 14% polyacrylamide separating gel. Electrophoretic transfer of the separated sample components from the SDS-PAGE gel to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.) was performed by the method of Towbin et al. (33). Subsequent steps of the lectin blotting procedure were conducted as reported by Millette and Scott (20). Both large and small molecular size standards (Sigma Chemical Co., St. Louis, Mo.) were simultaneously separated by gel electrophoresis and electrotransferred to nitrocellulose membranes under identical conditions. The standard polypeptide bands were visualized by incubation of the membrane with 0.5% Ponceau S stain (Sigma) in 0.1% acetic acid.

Isolation and purification of TP-Ags. The 120- and 110-kDa TP-Ags were isolated from the F+L fraction as described in our previous report (17). In brief, the sequential steps of purification included ConA affinity chromatographic separation of the crude F+L preparation, SDS-PAGE separation of the ConA-bound fraction under reducing conditions, electroelution of the 120- and 110-kDa bands from the preparative SDS-PAGE gels, and gel filtration or ion-exchange chromatographic separation of the electroeluted fractions, respectively. Homogeneity of the isolated 120- and 110-kDa fractions was demonstrated in silver-stained SDS-PAGE gels as described previously (17).

Deglycosylation of TP-Ags. Digestion of the carbohydrate moiety of the purified 120- and 110-kDa glycoproteins was performed essentially by the procedure of Cassone et al. (3). The purified and lyophilized TP-Ags (500 μg) were resolubilized in 500 μl of 0.2 M $NaIO_4$ in 0.05 M acetate buffer (pH 4.5) and incubated at 24°C for 18 h. The reaction was stopped by addition of an equal volume of spectrophotometric-grade ethylene glycol (Aldrich Chemical Co., Milwaukee, Wis.). The mixture was desalted and concentrated by ultrafiltration (Amicon) as described above, washed four times with HPLC-grade water (Fisher), and lyophilized. Approximately 100 μg of sample was recovered from each preparation. Periodate-treated samples were examined for immunoreactivity in the ID-TP assay and enzyme-linked immunosorbent assay (ELISA) as described below. The deglycosylated fractions were also subjected to SDS-PAGE (10% polyacrylamide gel) under reducing conditions and gas chromatography (GC)-mass spectroscopy (outlined below) for determination of protein content and to test for residual neutral sugar, respectively.

Monosaccharide composition of TP-Ags. The monosaccharide contents of the purified 110-kDa TP-Ag and the deglycosylated 120- and 110-kDa fractions were examined by GC-mass spectroscopy with authenticated, spectroscopic-grade sugar standards as previously described (6). The 3-*O*-methyl-D-mannose (3-*O*-MM) standard was synthesized and kindly provided by M. B. Goren.

Amino acid composition of 110-kDa TP-Ag. Approximately 500 μg of the purified 110-kDa TP-Ag was prepared for amino acid compositional analysis as previously described (6) with an autoanalyzer (model 420; Applied Biosystems, Foster City, Calif.).

ID assays. The immunodiffusion assays were performed by the method of Huppert and Bailey (14–16) for detection of anti-*C. immitis* TP or CF antibodies in sera from patients on the basis of their reactivity with the ID-TP or ID-CF reference antigens. The reference antigens and human reference sera were the same as reported earlier (8).

ELISA. ELISAs were conducted with an indirect screening kit (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) by the method previously reported (5) except that the peroxidase substrate used was 3,3′,5,5′-tetramethylbenzidine (U.S. Biochemicals, Cleveland, Ohio) and the intensity of the color reaction was determined by measuring the A_{450}. The reactivity of serum samples from 70 patients diagnosed with coccidioidomycosis (provided by M. L. Fried, Tucson Medical Center, Tucson, Ariz.; J. N. Galgiani, VA Medical Center, Tucson, Ariz.; T. N. Kirkland, University of California, San Diego, and A. Yi, Meridian Diagnostics Inc., Cincinnati, Ohio) and 70 control patients (provided by A. da Silva, Brackenridge Hospital, Austin, Tex.) with the 120- and 110-kDa TP-Ags was determined. All sera from patients with coccidioidomycosis used in this study were determined to be positive in the ID assay on the basis of detection of TP and/or CF antibodies to the corresponding *C. immitis* reference antigens (8). The control serum samples were obtained from patients admitted to the hospital with no recognized systemic or pulmonary mycoses. The purified 120- and 110-kDa fractions were examined in the ELISA at concentrations of 124 and 248 ng/ml of buffer, respectively. Optimal antigen concentrations were determined by block titration (17). A dilution of 1:400 of test sera (patient and control serum samples) in blocking solution was chosen for comparing reactivity of the coccidioidomycosis patient sera in the ELISA. Each serum sample from patients and controls was tested for binding of IgM and IgG antibodies to the purified 120- and 110-kDa fraction by using the respective specific goat anti-human immunoglobulin (goat anti-human IgG [γ-chain specific] and goat anti-human IgM [μ-chain specific]) conjugated with peroxidase (Kirkegaard & Perry) as previously described (6). Patient sera with optical density (OD) values higher than that of the mean of the control sera plus twice the standard deviation were considered positive (5). Assays with sera in the absence of antigen and with antigen in the absence of primary sera served as controls. All sera were tested in triplicate wells.

Sera from patients with other fungal diseases and mycobacterial infections were also tested in the ELISA for cross-reactivity of IgM and IgG antibodies with the same two purified TP-Ags of *C. immitis*. A separate set of serum samples from control patients was used in assays of each group of heterologous test sera. The test sera were obtained from 30 patients who were diagnosed with histoplasmosis (provided by L. Kaufman, Centers for Disease Control, Atlanta, Ga.; C. Cooper, New York Department of Health, Albany, L. J. Wheat, Wishard Memorial Hospital, Indianapolis, Ind.; and G. S. Kobayashi, Washington University School of Medicine, St. Louis, Mo.), 24 patients diagnosed with blastomycosis (provided by J. M. Jones, William S. Middleton Veterans Hospital, Madison, Wis.), 10 patients diagnosed with cryptococcosis and 9 patients diagnosed with systemic or mucocutaneous candidiasis (provided by M. Rinaldi, University of Texas Health Science Center, San Antonio), and 30 patients diagnosed with mycobacteriosis, including infections with *Mycobacterium tuberculosis*, *M. intracellulare*, *M. kansaii*, and *M. gordonae* (provided by M. B. Goren, National Jewish Hospital, Denver, Colo.). Additional serological data pertaining to these sera from patients with other mycoses and nonmycotic conditions were not available. All heterologous test sera and the separate set of control sera were diluted 1:200 in blocking solution for comparative purposes. This lower serum dilution was used to amplify potential cross-reactivity between heterologous patient sera and the purified TP-Ags.

IEP. The immunoelectrophoresis (IEP) procedure used combined zone electrophoresis with immunoprecipitation (19). Briefly, Gel Bond film (100 by 85 by 0.2 mm; FMC Corp., Rockland, Maine) was coated with 1% agarose (approximately 2 mm thick) prepared in barbital buffer (pH 8.6) as previously described (13). Wells (4-mm diameter) were cut in the agarose, and 15 μl of a pool of undiluted serum from five ID-TP-positive coccidioidomycosis patients or serum from control patients was added to the wells. The coccidioidomycosis patient sera which were chosen had ID-TP titers of 1.2 to 1.8. The serum components were separated by electrophoresis at 10 V/cm for 5 h at 5°C by using an LKB 2117 Multiphor II system (Pharmacia, Piscataway, N.J.). Troughs (65 by 1 mm) were then cut in the agarose on either side of the well, and antigen (125 μl of 120- or 110-kDa TP-Ag at a concentration of 10 μg/μl of PBS, pH 7.6) or antiserum (100 μl of either goat anti-human IgG [γ-chain specific] [Sigma] or goat anti-human IgM [μ-chain specific], diluted 1:4 in PBS) was added to the troughs. The agarose-coated film was subsequently incubated at 24°C for 18 h, washed in PBS, and stained with Coomassie brilliant blue R-250 (Sigma) to reveal the precipitin arcs (19).

Isolation of specific TP-Ag-reactive human antibody. Solid-phase immunoadsorption (SPIA) was used to isolate specific anti-120-kDa and anti-110-kDa antibody from the same pool of five ID-TP-positive patient serum samples used for the IEP assays described above. Antibody reactive with the 120-kDa TP-Ag was isolated by SPIA by using synthetic 3-*O*-MM conjugated with bovine serum albumin (BSA). The latter served as a ligand of the pseudoantigen for its immobilization to the solid phase. We previously demonstrated that coccidioidomycosis patient IgM antibodies have high affinity for the 3-*O*-MM-containing epitope(s) of the 120-kDa TP-Ag (6). The conjugated 3-*O*-MM was supplied by M. B. Goren and has been described elsewhere (12b). Coupling of the 3-*O*-MM–BSA conjugate to cyanogen bromide-activated Sepharose 4B (Pharmacia) and the subsequent washing, serum binding, and elution steps were the same as previously described (6). Antibody reactive with the 110-kDa TP-Ag was isolated separately from the same pool of patient sera by SPIA by using the purified 110-kDa fraction bound to cyanogen bromide-activated Sepharose. The eluate fractions (200 μl) from each affinity column were tested separately by the ELISA for reactivity with the homologous antigens as described above except that each antigen was used at a concentration of 250 ng/ml of buffer. Levels of serum binding were assayed by using goat anti-human IgG conjugated with peroxidase. The antibody fractions eluted from the 3-*O*-MM–BSA and 110-kDa TP-Ag affinity columns showing the highest OD values in the ELISA were concentrated separately by ultrafiltration (Amicon) as described above. The isolated human anti-110-kDa fraction was adsorbed with synthetic 3-*O*-MM (0.80 μg/ml of serum) at 4°C for 14 h (6) to minimize cross-reactivity with the 120-kDa TP-Ag.

IEM. The antigen-specific antibody fractions obtained by the methods described above were used separately for localization of each TP-Ag on thin sections of in vitro-grown spherules by immunoelectron microscopy (IEM) as previously reported (6). A range of dilutions of antibody fractions in blocking buffer (1:2 to 1:100) were tested for optimal binding to sections. The secondary antibody reaction mixture consisted of goat anti-human IgG conjugated with colloidal gold (15-nm-diameter particles; Sigma) plus 1% ovalbumin prepared in 0.02 M Tris-HCl buffer (pH 8.2). Thin sections that were reacted with human control sera and then with the secondary antibody-gold conjugate served as con-

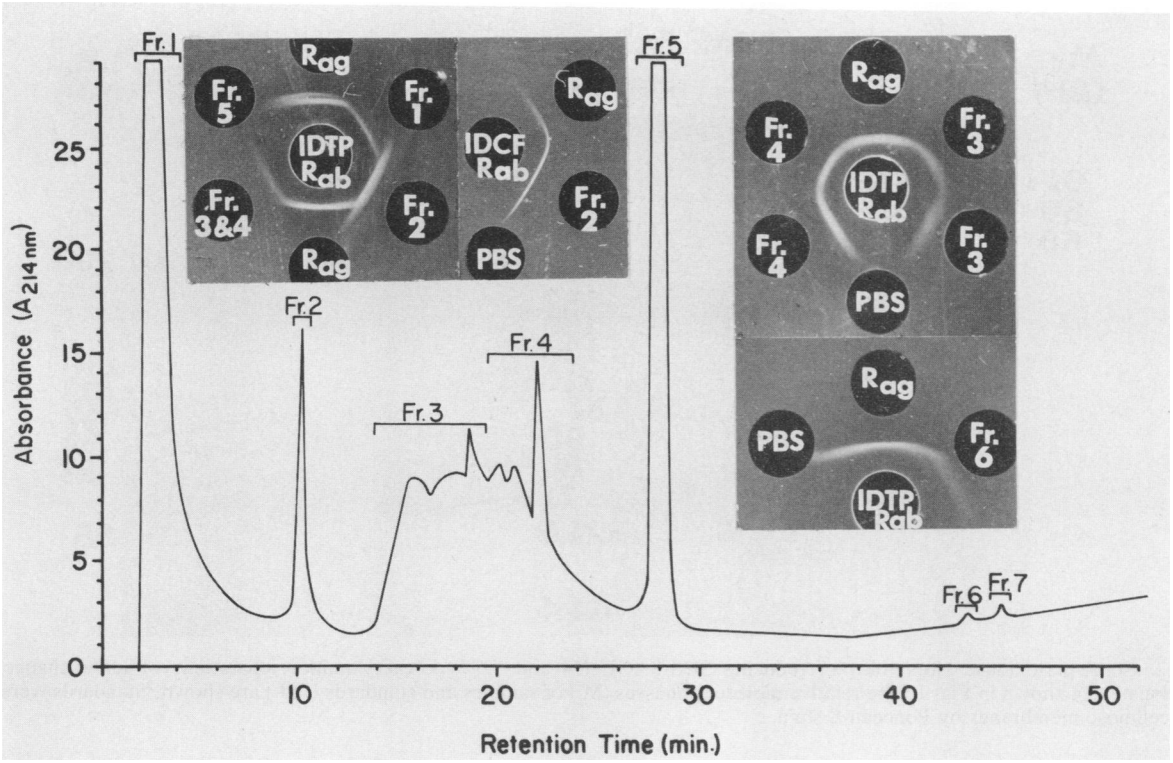

FIG. 1. Representative ion-exchange (DEAE) separation of the ConA-bound F+L fraction. Subfractions (Fr 1 to 7) were collected, concentrated, and tested for immunoreactivity in the ID-TP or ID-CF assay using appropriate reference antibodies (R_{ab}) and antigens (R_{ag}). Subfractions 3 and 4 were tested together and individually.

trols. Preparation of in vitro-grown spherules for thin sectioning involved techniques of cryofixation, freeze-substitution, and embedding in Spurr's low-viscosity resin (32) cured at 37°C for 96 h. For cryofixation, spherule suspensions were quick-frozen in Converse growth medium with an Aldrich-Erdos model QF-1000 propane jet freezer (AE Engineering, Gainesville, Fla.). Samples were freeze-substituted in one of two fixative solutions: (i) 1.0 ml of 70% glutaraldehyde (Polysciences, Warrington, Pa.) plus 0.2 ml of absolute acrolein (Polysciences) and 0.4 g uranyl acetate (Ted Pella, Tustin, Calif.) in 20 ml of anhydrous acetone (HPLC grade; Fisher) or (ii) 0.5 g of osmium textroxide (Electron Microscopy Sciences, Fort Washington, Pa.) plus 0.01 g of uranyl acetate dissolved in 22.5 ml of anhydrous acetone. Samples were maintained in the substitution solution for 48 h at −80°C and then transferred to −20°C for 24 h and −4°C for 2 h and brought to room temperature for 2 h. Samples were rinsed with three changes of fresh anhydrous acetone and then embedded in Spurr's resin. Thin sections were prepared, poststained, and examined as previously reported (6).

RESULTS

Identification of TP antibody-reactive subfractions of the ConA-bound F+L antigen complex. Figure 1 shows a representative elution profile of the ConA affinity-bound F+L sample fractionated by ion-exchange chromatography. Fractions 1 to 7 (Fr 1 to 7) were tested separately in the ID-TP assay, the results of which are also shown in Fig. 1. Frs 1, 3, 4, and 6 were ID-TP positive, Fr 2 produced a line of identity which showed only partial fusion with the TP reference precipitin line, and Frs 5 and 7 were not reactive in the ID-TP assay. Fr 2 was tested in the ID-CF assay and produced a line of identity which fused with the CF reference precipitin line. Results of the ConA blots of SDS-PAGE separations of each ID-TP-positive chromatographic fraction are shown in Fig. 2. Fr 1 probably represents material which failed to bind to the DEAE gel or demonstrated minimal affinity. Multiple bands were visible on the ConA blots of Fr 1 in the range of about 200 kDa (7.5% polyacrylamide gel) to 43 kDa (14% polyacrylamide gel). The ConA blots of Fr 2 showed lightly stained bands at 48 kDa (14% polyacrylamide gel) and 93 kDa (7.5% polyacrylamide gel), while blots of Frs 3 and 4 showed multiple bands in the range of 156 to 60 kDa. The blot of the 7.5% polyacrylamide gel of Fr 3 included the 120-kDa band, while the ConA blot of the 7.5% polyacrylamide gel of Fr 4 resolved both the 120- and 110-kDa bands. ConA blots of both 7.5 and 14% polyacrylamide gel separations of Fr 6 showed no visible bands on the nitrocellulose membrane.

ID assays of test sera. All serum samples from patients with coccidioidomycosis showed positive reactivity in the ID-TP and/or the ID-CF assay with standard reference reagents (8). None of the serum samples obtained from patients with other fungal or mycobacterial infections showed reactivity in either the ID-CF or ID-TP assay. All control sera were also negative in the ID assays.

Immunoreactivity and protein composition of the deglycosylated 120- and 110-kDa TP-Ags. Periodate oxidation of the purified 110-kDa fraction resulted in its loss of reactivity with precipitin antibody in sera from coccidioidomycosis patients as detected by the ID-TP assay (Fig. 3A). We had previously reported a loss of reactivity of the deglycosylated 120-kDa fraction when tested under the same conditions (6). The OD levels for IgM binding in the ELISA were compared by reacting the same set of five ID-TP-positive patient serum

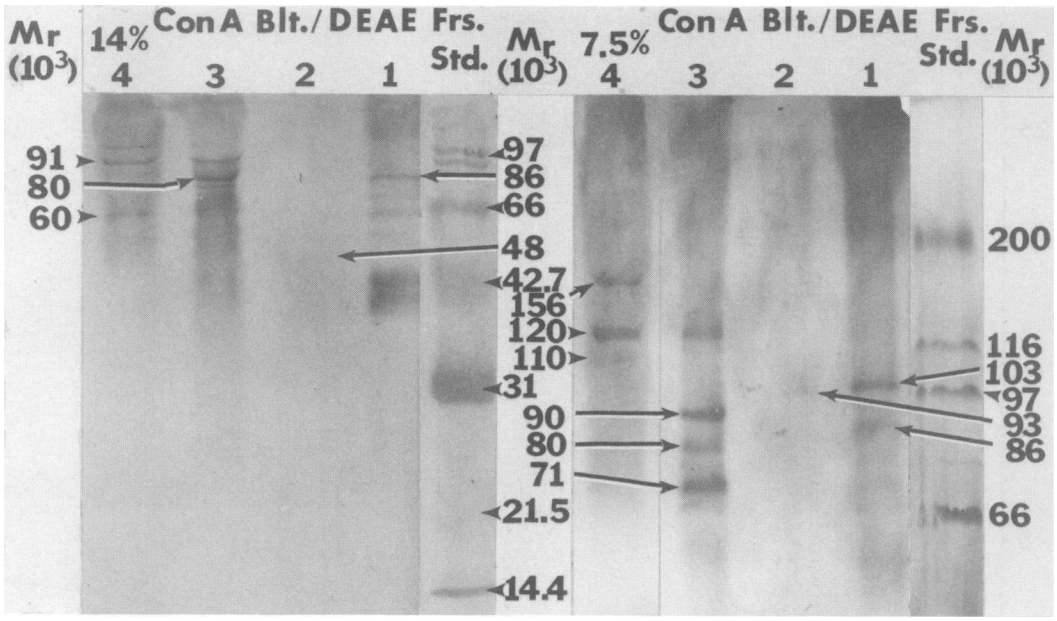

FIG. 2. ConA-perioxidase blots (blt.) of reducing SDS-PAGE (14 and 7.5% polyacrylamide) separations of ion-exchange (DEAE) subfractions 1 to 4 shown in Fig. 1. The relative molecular masses (M_r) of samples and standards (Std.) are shown. Standards were revealed on nitrocellulose membranes by Ponceau S stain.

samples described below for the IEP assay with the untreated or deglycosylated 120- and 110-kDa fractions. A loss of 82 to 95% of the patient IgM antibody binding was recognized. The average OD values for the reaction of patient sera with the untreated 120- and 110-kDa TP-Ags were 1.030±0.014 and 1.060±0.015, respectively. The average OD values for the reaction of the same patient sera with the deglycosylated 120- and 110-kDa TP-Ags were 0.173±0.014 and 0.052±0.008, respectively. When the $NaIO_4$-treated 120- and 110-kDa fractions were examined by SDS-PAGE under reducing conditions, 46- and 71-kDa polypeptide bands were revealed, respectively (Fig. 3B). The differences in estimated molecular size between the untreated and $NaIO_4$-treated 120- and 110-kDa TP-Ags were 74 and 39 kDa, respectively.

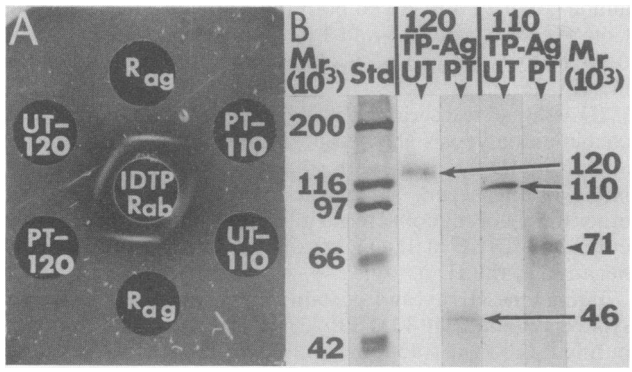

FIG. 3. (A) Results of ID-TP assay with reference antibody (R_{ab}) and antigen (R_{ag}) compared with results with sodium periodate-treated (PT) and untreated (UT) 120- and 110-kDa TP-Ags. (B) Reducing SDS-PAGE separation of purified UT and PT 120- and 110-kDa TP-Ags. The relative molecular masses (M_r) of samples and standards (Std) are shown.

Monosaccharide composition of the 120- and 110-kDa TP-Ags. The total removal of carbohydrate from the periodate-treated TP-Ags was confirmed by GC-mass spectroscopy (data not shown). A gas-liquid chromatogram of the purified, untreated 110-kDa fraction is shown in Fig. 4. The major component is mannose, and only a trace of glucose was detected. A comparison of the relative amounts of monosaccharides in the native 120-kDa (6) and 110-kDa fractions, based on integration of methylglycoside peaks of the GC, is presented in Table 1. Mannose is the major component of each fraction, while 3-*O*-MM, xylose, and galactose were detected only in the 120-kDa TP-Ag.

Amino acid composition of 110-kDa TP-Ag. The relative amounts of each amino acid of the 110-kDa fraction after hydrolysis and chromatographic analysis is presented in Table 2. The predominant amino acids were aspartic acid, serine, glutamic acid, and glycine. Cysteine was found only in a trace amount. The absence of tryptophan may have been due to its degradation during hydrolysis.

ELISA. The OD values obtained from the reaction of serum samples from 70 coccidioidomycosis patients with the 120- and 110-kDa TP-Ags are shown in Fig. 5 together with results of reactivity of control patient sera. We examined levels of both IgM and IgG binding for each serum sample to the purified antigenic fractions and have distinguished each sample in Fig. 5 on the basis of serological reactivity in the ID-TP and ID-CF assays (i.e., TP^+ CF^-, TP^+ CF^+, TP^- CF^+, or TP^- CF^-). Good correlation was revealed between results of the ELISA of IgM binding to the 120-kDa fraction and the ID-TP assays of patient sera (Fig. 5A). For example, 13 test serum samples determined to be TP^+ CF^- were all positive in the ELISA and showed a range of OD values for IgM binding to the 120-kDa TP-Ag of 0.201 to 1.253. The 36 test serum samples identified as TP^+ CF^+ were also all positive in the ELISA. In the case of the TP^- CF^+ serum samples, 14 of 21 showed negative OD values for IgM binding to the 120-kDa TP-Ag, and the majority of those

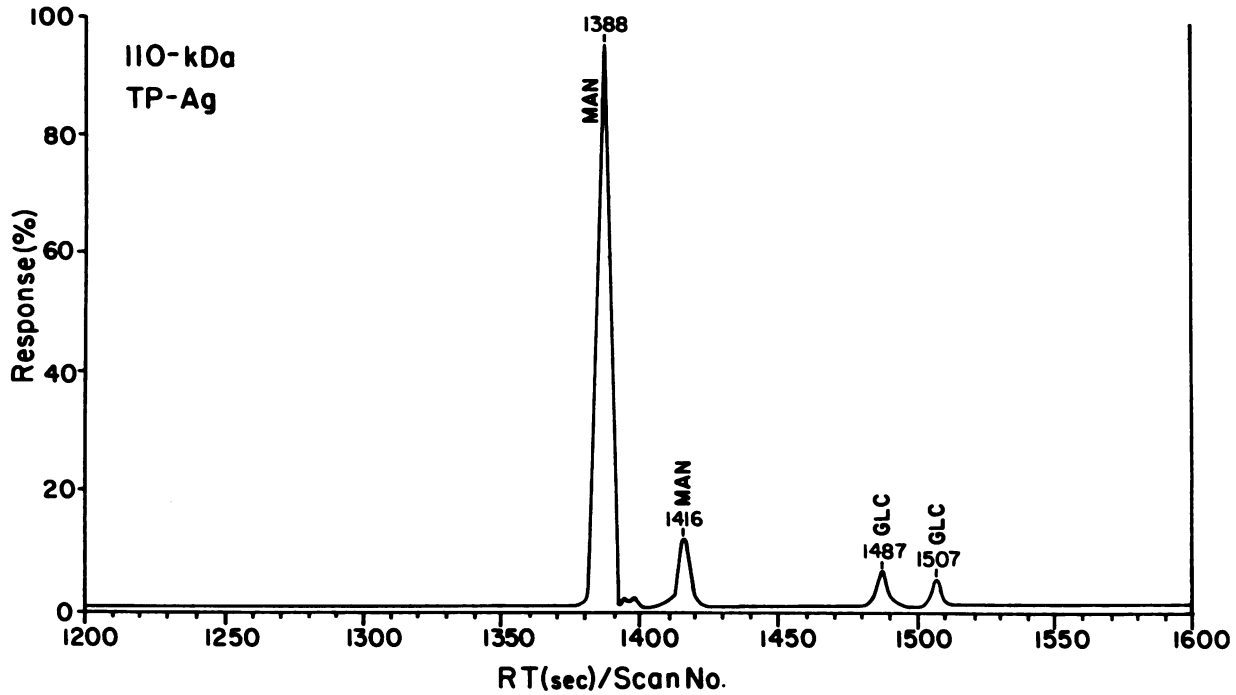

FIG. 4. Gas-liquid chromatogram of trimethylsilylated methylglycosides of monosaccharides present in the purified 110-kDa TP-Ag. Peaks are labeled as follows: MAN, mannose; GLC, glucose.

serum samples which were ELISA positive (6 of 7) revealed OD values close to the positive-negative cutoff line. All control sera (TP^- CF^-) diluted 1:400 in blocking solution showed uniformly low OD values. As indicated in a previous study (6), less correlation was evident between results of the ID assays and ELISAs of patient IgG antibody binding to the 120-kDa TP-Ag (Fig. 5A). For example, 19 of the 21 TP^- CF^+ serum samples demonstrated positive OD values, which is in contrast to results of IgM binding to the same fraction. Although all TP^+ CF^- and TP^+ CF^+ patient sera showed positive OD values or borderline positive/negative OD values for IgG binding to the 120-kDa fraction, the majority of sera in each case showed lower values than those revealed for IgM binding to the same fraction.

Comparison between OD values for patient IgM antibody binding to the 110-kDa TP-Ag and results of the ID assays (Fig. 5B) revealed that 12 of 13 TP^+ CF^- serum samples and 33 of 36 TP^+ CF^+ serum samples were also positive in the ELISA. The three TP^+ CF^+ serum samples which were negative in the ELISA were from patients with low OD values for IgM binding to the 120-kDa fraction shown in Fig. 5A. Correlation of OD values was demonstrated between IgM response of the same individual ELISA-positive patient sera to the 120-kDa (Fig. 5A) and 110-kDa (Fig. 5B) frac-

TABLE 1. Monosaccharide composition of 120- and 110-kDa TP-Ags

Monosaccharide	% of sugar content[a] of:	
	120-kDa TP-Ag	110-kDa TP-Ag
3-*O*-MM	7.5	ND
Xylose	7.6	ND
Mannose	66.5	94.0
Galactose	3.0	ND
Glucose	15.4	6.0

[a] The monosaccharide composition of chromatographically purified 120- and 110-kDa fractions (17) determined by GC analysis of trimethylsilyl methylglycosides of neutral sugars are expressed as percentages of total neutral sugar. ND, not detected.

TABLE 2. Amino acid composition of the 120- and 110-kDa TP-Ag isolated by gel filtration and ion-exchange chromatography[a]

Amino acid	% of total[b] in:	
	120-kDa TP-Ag[c]	110-kDa TP-Ag
Cysteine	4.74	0.46
Aspartic acid	8.90	12.50
Threonine	6.54	8.33
Serine	10.25	9.02
Glutamic acid	13.28	11.43
Proline	4.94	6.26
Glycine	14.77	10.88
Alanine	6.41	8.59
Valine	4.61	4.78
Methionine	1.05	1.26
Isoleucine	3.19	4.52
Leucine	6.52	5.12
Tyrosine	0.98	1.61
Phenylalanine	3.26	2.88
Lysine	5.41	3.86
Histidine	1.60	1.37
Arginine	3.48	3.18
Tryptophan	ND	ND

[a] The 120- and 110-kDa TP-Ags were isolated by gel filtration and ion-exchange (DEAE) chromatography, respectively, as previously described (17).
[b] Values are expressed as percentages of total nanomoles of amino acids detected. ND, not detected.
[c] From reference 6.

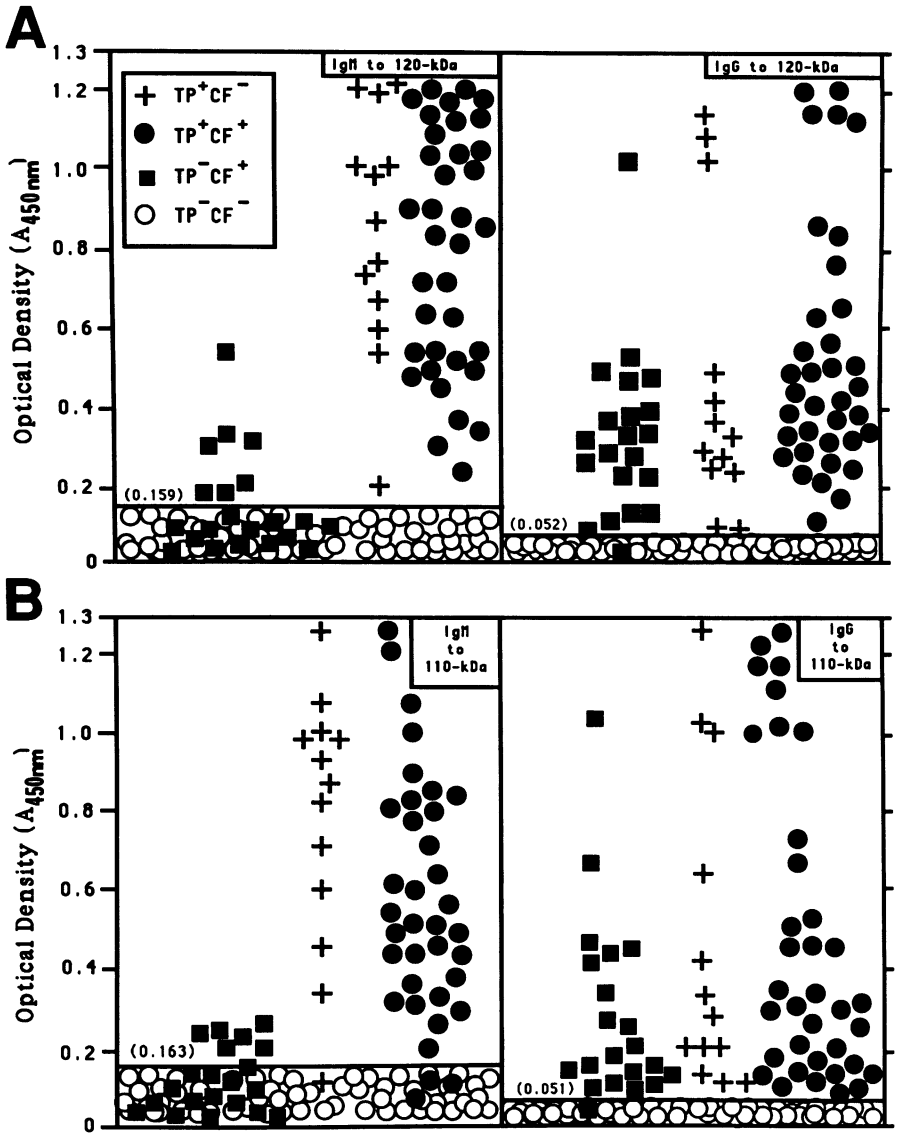

FIG. 5. Results of ELISAs with control serum samples (○) and serum samples from three categories of patients with coccidioidomycosis adsorbed to either the purified 120-kDa TP-Ag (124 ng/ml) (A) or 110-kDa TP-Ag (248 nm/ml) (B) bound to wells of microtitration plates. The categories of patient sera used are distinguished by reactivity in the ID-TP and ID-CF assays. Goat anti-human IgM and goat anti-human IgG conjugated with peroxidase were used for detection of adsorbed antibody. The values for the mean OD of the control sera plus twice the standard deviation are shown above the solid lines. Control and patient serum samples were diluted 1:400 in blocking solution.

tions. Of the 21 TP^- CF^+ serum samples, 15 were negative in the ELISA when IgM binding to the 110-kDa fraction was examined, and the 6 ELISA-positive serum samples correlated with positive OD values for IgM binding to the 120-kDa TP-Ag. As demonstrated by results of IgG binding to the 120-kDa fraction, an apparent lack of correlation between results of ID assays and IgG antibody binding to the 110-kDa fraction is also revealed in Fig. 5B. Only 1 of the 21 TP^- CF^+ serum samples was also negative in the ELISA of IgG response to the 110-kDa fraction. This same TP^- CF^+ patient serum sample was negative in the ELISA for IgG binding to the 120-kDa fraction.

Results of analyses of cross-reactivity between sera from patients with other fungal diseases or mycobacterial infections and the isolated 120- and 110-kDa TP-Ags are presented in Fig. 6. The five groups of heterologous test sera consisted of 109 samples. Of these, only 3 (2.8%) showed IgM cross-reactivity with the 120-kDa fraction in the ELISA (Fig. 6A). One of the samples was derived from a patient with histoplasmosis, and the other two were from patients with oral candidiasis (thrush). It was not known whether the former sample was from a case of primary histoplasmosis (21). These three serum samples showed no reactivity in the ID-TP or ID-CF assays with the *C. immitis* reference antigens. When levels of adsorbed IgM antibody from the same groups of heterologous sera were examined by the indirect ELISA using the 110-kDa fraction, 23 of 109 samples (21.1%) showed positive OD values (Fig. 6B). The three serum samples which were positive in the ELISA for IgM binding to the 120-kDa fraction were also positive for IgM

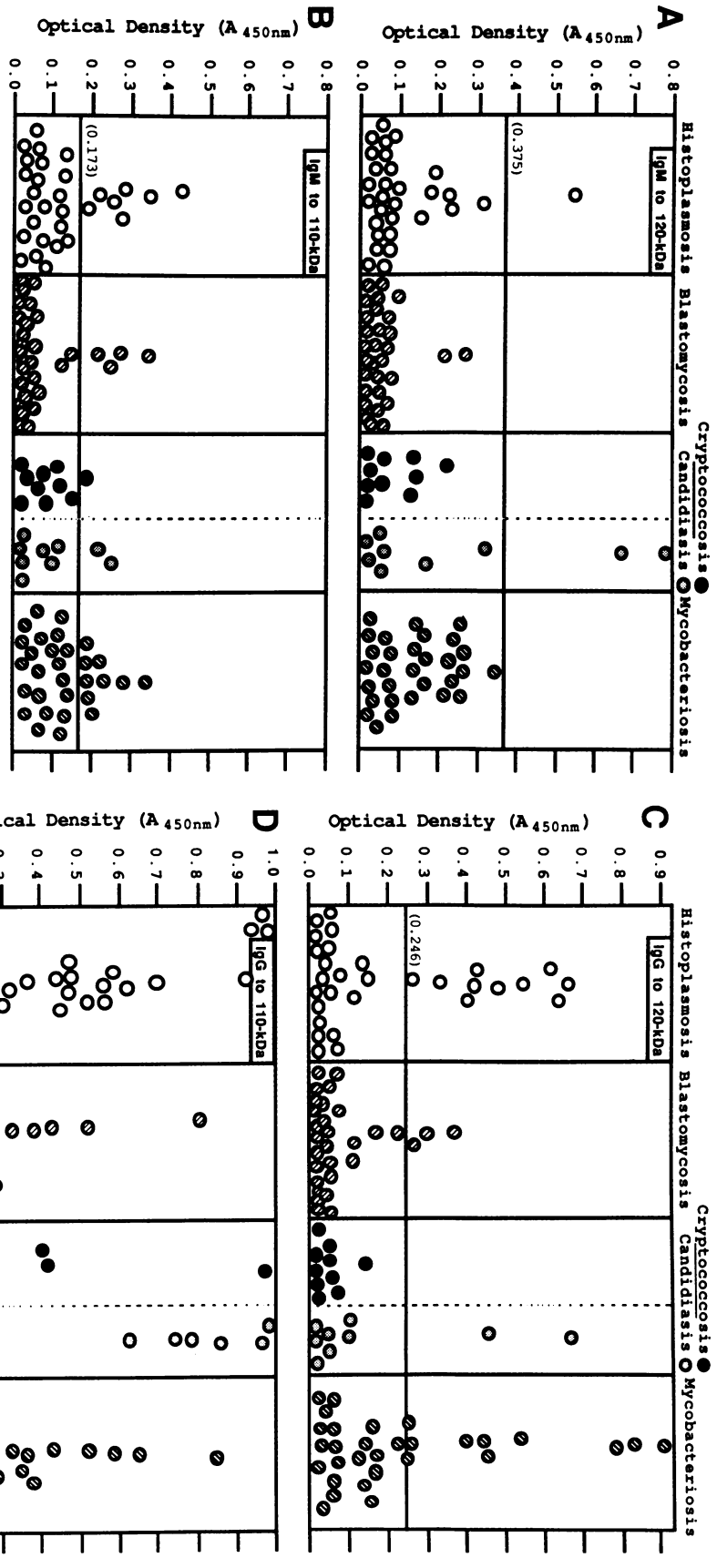

FIG. 6. Results of ELISAs with serum samples from patients with histoplasmosis, blastomycosis, cryptococcosis, candidiasis, and mycobacteriosis adsorbed to either the purified 120-kDa TP-Ag (124 ng/ml) (A and C) or 110-kDa TP-Ag (248 ng/ml) (B and D). The secondary antibody conjugates were the same as those described in the legend to Fig. 5. Results of ELISAs with control serum samples are not shown. The values for the control sera plus twice the standard deviation are shown above the solid lines. Control and patient serum samples were diluted 1:200 in blocking solution.

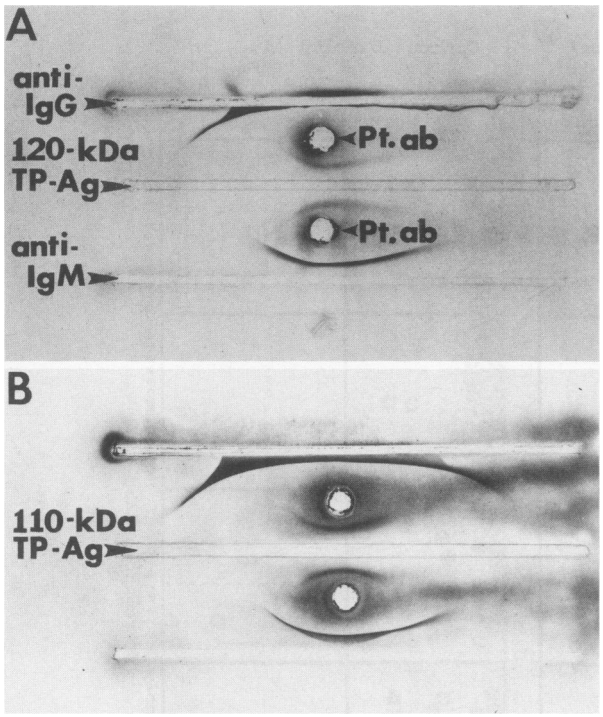

FIG. 7. Results of IEP assays with a pool of sera from patients with coccidioidomycosis (Pt. ab) added to the wells and either the purified 120-kDa TP-Ag (A) or 110-kDa TP-Ag (B) (10 μg/μl) added to the central trough. The upper and lower troughs in each panel contained goat anti-human IgG and goat anti-human IgM, respectively.

binding to the 110-kDa fraction. High levels of cross-reactivity between IgG antibody from all groups of heterologous sera and the 120-kDa fraction (70.6%) (Fig. 6C) and 110-kDa fraction (68.8%) (Fig. 6D) were demonstrated.

IEP assays. Results of the IEP assays of ID-TP-positive coccidioidomycosis patient serum reactivity with the purified 120- and 110-kDa TP-Ags are shown in Fig. 7A and B, respectively. In each case, a single precipitin arc was formed adjacent to the trough containing the TP-Ag. Its location corresponded to that of the reference anti-IgM precipitin.

Immunolocalization of the 120- and 110-kDa TP-Ags. Since no 3-*O*-MM was detected in the 110-kDa fraction, it was logical to use the synthetic 3-*O*-MM–BSA conjugate to isolate anti-120-kDa antibody from coccidioidomycosis patient sera by SPIA on the basis of the previously demonstrated high affinity of patient antibody for this sugar-containing epitope (6). However, some cross-reactivity between the anti-120-kDa serum eluate and the 110-kDa fraction was observed by the ELISA. The OD values resulting from reaction of the eluate derived from the 3-*O*-MM–BSA-containing SPIA column with the 120 kDa TP-Ag was 3.311 ± 0.028, while reactivity of the same patient serum fraction with the 110-kDa TP-Ag was 0.419 ± 0.018. This anti-120-kDa serum fraction was used for IEM at a dilution of 1:100 in buffer. The anti-110-kDa patient serum fraction obtained by SPIA and adsorbed with 3-*O*-MM before it was tested in the ELISA showed little cross-reactivity with the 120-kDa TP-Ag. The OD of the preadsorbed anti-110-kDa eluate reacted with the homologous antigen was 1.475 ± 0.070, while the OD of the same serum preparation reacted with the 120-kDa fraction was 0.039 ± 0.005. This anti-110-kDa patient serum fraction was used for IEM at a dilution of 1:50.

A minimal amount of gold label was visible on the wall of presegmented spherules which were reacted with patient control serum (Fig. 8A). Reaction between the affinity-isolated anti-120-kDa fraction and spherule sections revealed label localized in cytoplasmic vesicles and in the region of the plasma membrane and randomly distributed gold particles at lower concentrations on the spherule wall (Fig. 8B). Reaction between the preadsorbed, affinity-isolated anti-110-kDa serum fraction and spherule sections showed localization of gold particles within the same cytoplasmic vesicles (Fig. 9) and on the spherule wall. However, additional label was visible at the cell surface in association with the sloughing spherule outer wall (5, 8).

DISCUSSION

Evidence has been presented in this paper that detection of IgM antibody in human sera to a 120-kDa glycoprotein of *C. immitis* by indirect ELISA is a reliable serologic test for diagnosis of early stages of coccidioidal infection. We previously demonstrated that the 120-kDa fraction elicits a positive reaction in the ID-TP assay using sera of coccidioidomycosis patients (17). We have shown that the patient precipitin antibody reactive with the purified 120-kDa glycoprotein is of the IgM class, which is in agreement with results of earlier studies of TP antibody-reactive fractions of *C. immitis* (22, 24, 29). The clinical significance of detection of this antibody is that about 90% of individuals who have contracted primary, nondisseminating coccidioidomycosis produce IgM precipitin antibody to *C. immitis* antigen, as detected by the TP test (31). The high sensitivity of IgM antibody detection by the ELISA is an advantage over the conventional latex agglutination and ID assays currently used for diagnosis of early infection by *C. immitis* (35). Low titers of anti-120-kDa IgM which are not detected by other conventional methods may be detected by the ELISA. In spite of the difference in levels of sensitivity, however, good correlation was demonstrated between results of the ID-TP assay and ELISA of IgM response to the 120-kDa fraction in 70 serum samples from proved coccidioidomycosis patients and 70 control serum samples examined in this study. These results are in agreement with preliminary data presented by others which suggested that good correlation exists between these two methods of detection of *C. immitis*-specific IgM antibody (24). The lack of correlation between certain ID-TP-negative (ID-CF-positive) sera and the ELISA of IgM binding to the 120-kDa fraction can be explained largely on the basis of the higher sensitivity of the ELISA for detection of reactive antibody. Another factor, however, is that detection of levels of antibody binding to the 120-kDa fraction by the indirect ELISA using anti-IgM (γ-chain-specific) secondary antibody conjugate accounts for total bound, antigen-specific IgM and not precipitin antibody alone. It is possible, therefore, that occurrence of positive OD values in the ELISA with certain ID-TP-negative (ID-CF-positive) patient sera reflects elevated levels of anti-120-kDa IgM other than precipitin IgM antibody. All TP^- CF^- (control) sera showed comparatively low OD values in the ELISA. No false positives were found among this group of patient serum samples. An overall comparison of results of the ID-TP assay and the ELISA of IgM binding to the purified 120-kDa fraction in this study revealed that 95% (133 of 140) of the tests agreed.

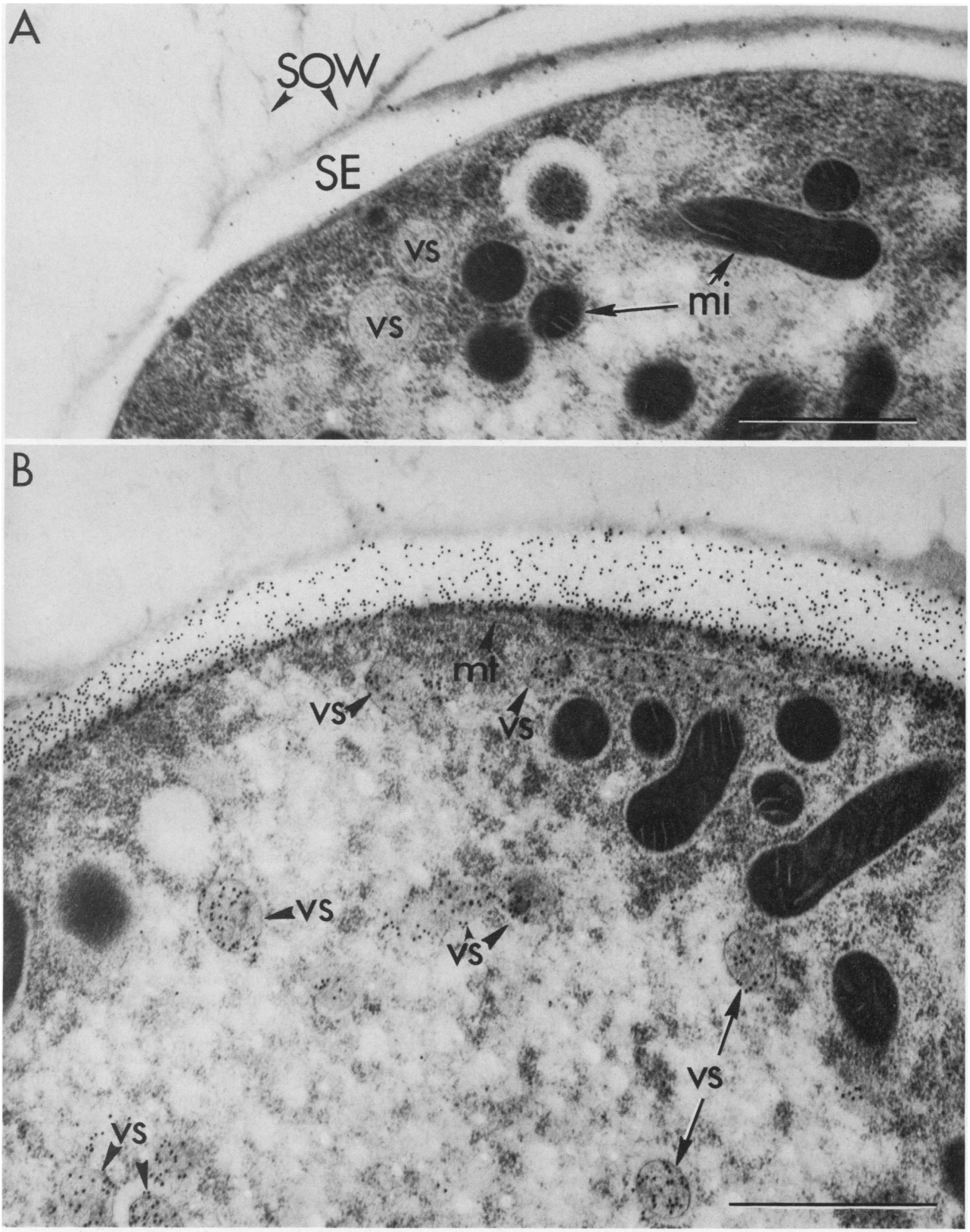

FIG. 8. Thin sections of presegmented spherules showing distribution of human control serum (A) or affinity-isolated human anti-120-kDa TP-Ag–gold conjugate immunolabel (B). Abbreviations: mi, mitochondria; mt, microtubule, SE, spherule envelope; SOW, spherule outer wall; vs, vesicle. Bars, 1.0 μm.

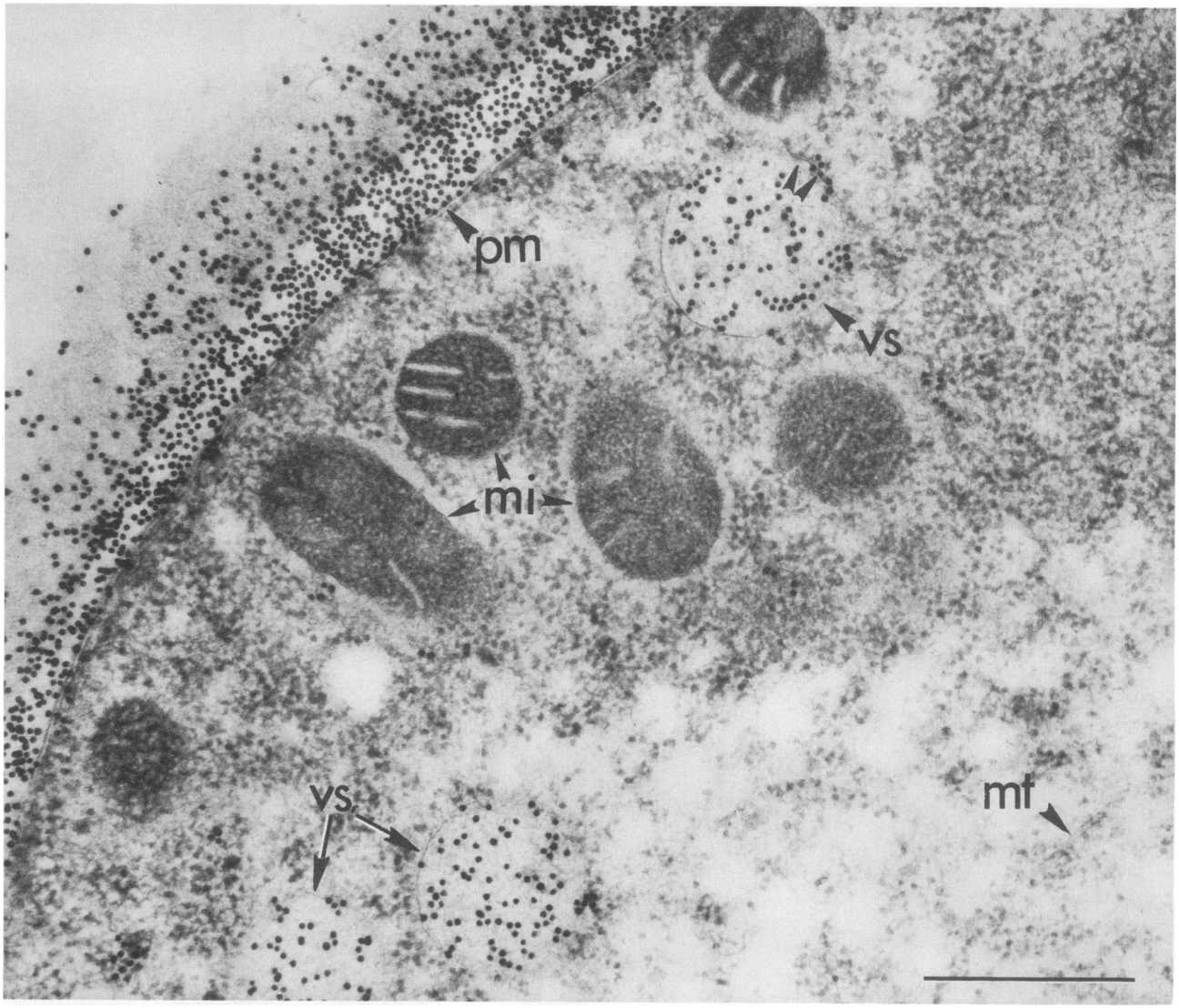

FIG. 9. Thin section of presegmented spherule showing distribution of affinity-isolated human anti-110-kDa TP-Ag–gold conjugate immunolabel. Abbreviations: mi, mitochondria; mt, microtubule; pm, plasma membrane; vs, vesicles. Unlabeled arrowheads locate a tripartite portion of vesicle membrane. Note gold immunolabel on the inner layer of the vesicle membranes and outer layer of the plasma membrane. Bar, 0.5 μm.

The specificity of the ELISA using the purified 120-kDa fraction for detection of reactive IgM in sera from patients with coccidioidomycosis was tested with 109 serum samples from proved cases of other mycoses as well as mycobacterial infections. This latter group was included because of the reported serological cross-reactivity between *C. immitis* antigens and tuberculin (28). An equal number of control serum samples were also examined. Cross-reactivity was examined with a serum dilution of 1:200 in blocking buffer (Fig. 6), twice the serum concentration used in the comparative ELISAs of test sera from patients with proved coccidioidomycosis (Fig. 4), in order to amplify potential reactions between heterologous patient sera and the purified TP-Ags. This accounts for the higher mean OD value for the control sera in Fig. 6. Only 2.8% (3 of 109) of the heterologous patient serum samples showed IgM cross-reactivity with the purified 120-kDa fraction.

Earlier reports of *C. immitis* antigens capable of eliciting a TP antibody response in patients identified the reactive macromolecules by a wide range of molecular sizes (21 to 330 kDa) (2, 11, 12, 37). The ConA-bound material of the F+L fraction was previously shown to contain both the 120- and 110-kDa TP-Ags (17). Separation of the lectin-bound F+L material by ion-exchange chromatography yielded four subfractions with ID-TP activity and one which was positive in the ID-CF assay. The 120- and 110-kDa glycoproteins were revealed as prominent components of two ID-TP-positive DEAE fractions. We showed that the ID-CF-positive subfraction contained a 48-kDa glycoprotein with apparently weak affinity for ConA. Zimmer and Pappagianis (36) isolated and purified an antigen with ID-CF activity from the spherule-endospore culture filtrate of *C. immitis* which they identified as a 48-kDa fraction by SDS-PAGE under reducing conditions. A 21-kDa serine proteinase was also isolated

from this same culture filtrate (26) and shown to have ID-TP activity (27). We previously isolated a 19-kDa serine proteinase from both the soluble conidial wall fraction and mycelial culture filtrate of *C. immitis* (7, 9) and have shown that its N-terminal amino acid sequence is partially identical to that of the 21-kDa proteinase (4). We have determined that the nonreduced form of this serine proteinase isolated from the mycelial culture filtrate has ID-TP activity but that the reduced form does not (unpublished data). We have also demonstrated that the nonreduced form of the proteinase binds readily to ConA while the boiled and β-mercaptoethanol-treated 19-kDa fraction has very low affinity for the lectin (7). This would account for the absence of a 19-kDa band in lectin blots of the DEAE fractions which were separated by SDS-PAGE under reducing conditions. One of the minor fractions with apparently high affinity for DEAE (i.e., Fr 6) was positive in the ID-TP assay. The composition of this fraction was not determined. The ConA-bound F+L material of *C. immitis* contains several distinct immunoreactive macromolecules which are recognized in both the ID-TP and ID-CF assays. The former are apparently components of an antigen complex of *C. immitis* which elicit a TP antibody response in patients.

We focused our attention on characterization of two isolated fractions of the TP antigen complex, namely, the 120- and 110-kDa TP-Ags. After establishing that the 120-kDa fraction was a potentially valuable reagent for diagnosis of primary coccidioidomycosis, we similarly evaluated the immunodiagnostic potential of the purified 110-kDa TP-Ag and compared its composition and immunolocalization in spherules with results of earlier investigations of the 120-kDa TP-Ag (6, 17). Patient precipitin antibody reactive with the purified 110-kDa antigen is also of the IgM class. Good correlation was also evident between results of ID assays and ELISAs of IgM but not IgG binding to the 110-kDa TP-Ag. As expected, sera from patients presumably in early stages of coccidioidal infection (i.e., TP^+ CF^- and TP^+ CF^+) showed higher levels of IgM than IgG antibody binding to both the 120- and 110-kDa fractions in the ELISA, while the opposite was observed with sera from patients with disseminated disease (i.e., TP^- CF^+). The IgM precipitin antibody response in patients with coccidioidomycosis is known to be short-lived, typically disappearing within 6 months (21, 24). Detection of IgG binding to the 120- and 110-kDa TP-Ags in almost all patient sera identified as TP^- CF^+ suggests that IgG response to these antigens persists even after precipitin antibody reactivity has disappeared. However, detection of IgG binding to the TP-Ags was determined to be of little value for diagnosis of coccidioidomycosis because of high levels of cross-reactivity between IgG in heterologous sera and both the 120- and 110-kDa fractions as detected in the ELISA. We have shown that in approximately 70% of the sera from patients with histoplasmosis, blastomycosis, cryptococcosis, candidiasis, and mycobacterial infections, IgG antibody cross-reacted with both the purified 120- and 110-kDa fractions. More important, IgM antibody in approximately 21% of these same heterologous sera showed cross-reactivity with the 110-kDa TP-Ag. This was a surprising observation in light of the comparable response of IgM in sera from coccidioidomycosis patients to both the 120-kDa and 110-kDa fractions as detected by both the ELISA and ID-TP assay.

To explore the possible basis of the observed difference in patient IgM specificity for the 120- and 110-kDa fractions, we examined the carbohydrate composition of the two TP-Ags. After periodate oxidation, both antigens lost their ability to elicit a precipitin antibody response in the ID-TP assay. Approximately 82 to 90% loss of IgM binding to the deglycosylated antigens occurred compared with IgM response to the native antigens as detected by the ELISA. Results of SDS-PAGE separation of the $NaIO_4$-treated fractions suggested that the 120-kDa TP-Ag was more glycosylated than the 110-kDa TP-Ag. Comparison of the monosaccharide content of the 120-kDa glycoprotein (6) and 110-kDa fraction by GC-MS analysis revealed that xylose, 3-*O*-MM, and galactose were present in the former but not the latter. We presented evidence earlier, (6) based on results of competitive inhibition ELISAs, that 3-*O*-MM residues associated with the 120-kDa fraction are at least partly responsible for reactivity of IgM antibody with this TP-Ag. Since 3-*O*-MM is apparently unique to *C. immitis* among the systemic fungal pathogens (6, 34), we suggest that this sugar may contribute significantly to the specificity of adsorption of IgM antibody to the 120-kDa TP-Ag. Isolation and structural analysis of the 120-kDa carbohydrate fraction are necessary to further examine its role in the immunospecificity of this TP-Ag.

The amino acid composition of the 120-kDa glycoprotein (6) and the purified 110-kDa fraction demonstrated certain similarities. Both showed Asp, Ser, Glu, and Gly as the predominant amino acids. On the other hand, the amount of Cys in the 120-kDa fraction was 4.74% of the total amino acids detected, while only a trace of Cys was present in the 110-kDa TP-Ag. We suggested earlier (6) that cysteine in the 120-kDa fraction may be associated with formation of disulfide bonds, which would account for the presence of both the monomeric (120-kDa) and dimeric (240-kDa) forms of this TP-Ag which were detected by gel filtration. No such dimerization of the purified 110-kDa fraction was recognized when the electroeluted, DEAE-purified glycoprotein was subjected to gel filtration chromatography. The results of IEM showed that the two TP-Ags are colocalized in cytoplasmic vesicles within presegmented spherules. Both glycoproteins are probably transported and perhaps processed within these organelles prior to their release to the cell wall upon fusion of the vesicles with the plasmalemma. The IEM data suggest a different distribution of the two TP-Ags within the spherule wall. The 120-kDa antigen is concentrated in the region of the plasmalemma and distributed at lower concentrations throughout the spherule wall. The 110-kDa antigen is concentrated adjacent to the outer surface of the plasmalemma (periplasmic space?), within the spherule wall, and in association with the outermost, sloughing, membranous spherule wall material (8). In support of this last observation, we have detected high concentrations of the 110-kDa fraction in the culture filtrate of preendosporulating spherules. An abundance of TP-antibody-reactive 120-kDa fraction was obtained by alkali extraction of the isolated spherule wall (unpublished data). The 110-kDa TP-Ag may be a secreted glycoprotein while the 120-kDa fraction remains associated with the cell wall during spherule development and is then naturally released at the time of endosporulation. Current investigation of the functions of these glycoproteins in *C. immitis* morphogenesis may provide clues about the nature of their interrelationship during the parasitic cycle.

ACKNOWLEDGMENT

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