INFECTION AND IMMUNITY, Jan. 1990, p. 169–178
0019-9567/90/010169-10$02.00/0

Vol. 58, No. 1

Isolation of Tube Precipitin Antibody-Reactive Fractions of *Coccidioides immitis*

DAVID KRUSE AND GARRY T. COLE*

Department of Botany, University of Texas, Austin, Texas 78713-7640

Received 6 April 1989/Accepted 4 October 1989

Patients presenting with primary coccidioidal infection have been shown by earlier investigators to produce immunoglobulin M (IgM) precipitin antibodies to lysates of mycelial and spherule phases of *Coccidioides immitis*. This humoral response has been detected by tube precipitin (TP) and immunodiffusion (ID)-TP assays of patient sera, which are valuable aids in early diagnosis of coccidioidomycosis. Several reports of antigenic fractions which show reactivity with patient TP antibody have been published. However, confusion persists with respect to the nature of the specific serologically reactive macromolecule(s). In this study we isolated two TP antibody-reactive antigens (TP-Ags) from an alkali-soluble, water-soluble fraction of the inner conidial wall and a culture filtrate plus toluene lysate of the mycelial phase of *C. immitis*. The crude antigens were first separated by concanavalin A (ConA) chromatography. The TP-Ags were identified in ID-TP assays as 120- and 110-kilodalton (kDa) fractions which were electroeluted from reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis separations of the ConA-bound conidial wall extract and ConA-bound culture filtrate plus lysate preparation, respectively. Following electroelution, the 120-kDa fraction was subjected to gel filtration chromatography which yielded a major 240-kDa and minor 120-kDa component. The apparent dimer may be a product of disulfide bond formation resulting from reassociation of the reduced, monomeric components (120 kDa). The latter was suggested by the presence of cysteine in the isolated fraction. The electroeluted 110-kDa fraction was subjected to ion-exchange chromatography. The DEAE-isolated, TP antibody-reactive fraction was identified as antigen 2 in the coccidioidin–anti-coccidioidin reference system. Homogeneity of the TP-Ags was demonstrated in silver-stained sodium dodecyl sulfate-polyacrylamide gels of the respective chromatographically isolated fractions. The two purified TP-Ags showed reactivity in the TP and ID-TP assays and were capable of binding patient IgM but comparatively little IgG antibody, as determined by an enzyme-linked immunosorbent assay. It appears that the diagnostic TP reaction between sera from patients with coccidioidomycosis and the ID reference antigens examined in this study is a composite of IgM binding to both a 120-kDa and a 110-kDa antigen.

Coccidioidomycosis is a respiratory disease of humans caused by inhalation of dry, airborne arthroconidia of the soil-inhabiting fungus *Coccidioides immitis* (14). Although the majority of infections are subclinical and resolve spontaneously, *C. immitis* is recognized as a primary pathogen capable of dissemination from the lungs to almost all organs of the body (25). Diagnosis of this fungal disease depends primarily on serologic tests (1, 2, 9). The majority of patients presenting with primary coccidioidal infection produce anti-*Coccidioides* immunoglobulin M (IgM) precipitin antibodies. Patient antibodies have been detected by reacting sera with coccidioidin (CDN), a lysate of the mycelial phase of *C. immitis*, in the classical tube precipitin (TP) test (22, 26–28). This serologic test has been replaced by an immunodiffusion (ID)-TP assay (19) in which precipitin antibodies have been detected adsorbed to antigens derived from both the saprobic- and parasitic-phase culture filtrates plus toluene lysates of the fungal cell mats, an autolysate of spherules grown in vitro (i.e., spherulin), and an alkali-soluble, water-soluble (ASWS) fraction of mycelial or spherule walls (9–11, 15). Detection of patient antibody to the TP antigen is a valuable aid in diagnosis of early coccidioidal infection. However, our knowledge of the nature of the specific antigen(s) responsible for this serologic reactivity is still incomplete (9, 10, 15, 32). We previously described the fractionation of an ASWS cell wall extract of *C. immitis* by lectin affinity chromatography followed by gel filtration, which yielded a methylated mannose polysaccharide-containing fraction with reactivity in the ID-TP assay (D. Kruse, J. W. Chinn, L. M. Pope, and R. W. Wheat, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, F52, p. 400). Details of the purification and characterization of IgM precipitin antibody-reactive fractions isolated from both an ASWS extract of the conidial wall and a culture filtrate plus toluene lysate of the mycelial phase of *C. immitis* are reported here.

MATERIALS AND METHODS

Cultivation. Arthroconidia of *C. immitis* 735 (isolated from a patient with disseminated coccidioidomycosis) were produced in plate culture on glucose-yeast extract agar and isolated by vacuum harvesting (5).

Isolation of crude antigenic fractions. Two crude antigenic fractions were examined. One was an alkali extract of the inner conidial wall fraction (ICWF), which was isolated as described previously (7). Extraction of the lyophilized ICWF was performed with 1 N NaOH by a modification of the method of Cox et al. (11). The dried cell wall material was suspended in freshly prepared 1 N NaOH (1 mg of sample per ml), agitated for 3 h at room temperature on a wrist-action shaker, and centrifuged (27,000 × *g*, 20 min, 4°C). The supernatant was then dialyzed against distilled water (cellulose dialysis tubing; molecular weight cutoff, 12,000 to 14,000; Medical Industries Inc., Los Angeles, Calif.) at 4°C for 72 h (six changes of dialysate, 6 liters each), and the retained material was lyophilized. The alkali-solubilized subfraction of the ICWF (ICWF-ASWS) was stored at

* Corresponding author.

−20°C. The second crude antigenic fraction used in this study was a mycelial culture filtrate plus toluene lysate, designated as the ID-TP reference antigen (75F+L). This crude fraction was obtained from a pool of 24 strains of *C. immitis* by the method described by Huppert and Bailey (18). This same dialyzed and lyophilized filtrate plus lysate preparation was used as a reference antigen for the ID tests described below.

ID. ID tests were performed by the method of Huppert and Bailey (17–19) for detection of antigens which reacted with TP or complement fixation (CF) antibodies of reference sera from patients with coccidioidomycosis. The ID-TP reference antigen was prepared as described above. The ID-CF reference antigen and the human reference sera were the same as previously described (4). All fractions examined in this study were also tested for reactivity in the *Coccidioides*-TP and -CF Immunodiffusion Systems (Meridian Diagnostics, Inc., Cincinnati, Ohio) according to the directions of the manufacturer. ID plates were incubated at room temperature for 72 h and examined daily for lines of identity with the reference antigens.

Gel electrophoresis, ConA blotting, and immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was conducted under reducing conditions by using either a 7.5 or 14% separating gel with a 5% stacking gel and the discontinuous buffer system described by Laemmli (20). Both low-molecular-size standards (14.4 to 97.4 kilodaltons [kDa]) and high-molecular-size standards (42.7 to 200 kDa) were used for calibration (Bio-Rad Laboratories, Richmond, Calif.). Gel components were revealed by using Coomassie blue R-250 (Sigma Chemical Co., St. Louis, Mo.) or by a silver staining procedure (23). Samples were heated for 5 min at 100°C in sample buffer (30) which contained 2% SDS and 0.4 M 2-mercaptoethanol. Other details of the SDS-PAGE procedure have been reported previously (30). Electrophoretic transfer of proteins and glycoproteins from the SDS-PAGE gel to membranes (Immobilon; Millipore Corp., Bedford, Mass.) was performed by the method of Towbin et al. (29). After transfer, the membrane was incubated in blocking solution (1.0 mM Tris hydrochloride buffer [pH 7.4] containing 0.9% [wt/vol] NaCl, 3% [wt/vol] bovine serum albumin [Sigma], and 0.005% thimerosal [Sigma]) for 2 h at room temperature with gentle agitation. Subsequent steps of the lectin blotting procedure were conducted as reported by Millette and Scott (21). The Immobilon membrane was incubated with 2 μg of concanavalin A (ConA)-peroxidase (Sigma) per ml in blocking solution for 2 h at room temperature. The immunoblotting procedure was the same as reported previously (8). ID-TP-positive patient sera were used at a 1:200 dilution in blocking solution. After being washed, the Immobilon membrane was incubated with goat anti-human IgM conjugated to peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) at a 1:200 dilution in blocking solution. Immobilon membranes to which glycoproteins were transferred and then reacted with human control serum samples (as described above for the TP assay) or secondary antibody-conjugate alone served as controls. Blots were developed in a solution containing 4-chloro-1-naphthol (Kirkegaard & Perry).

Antigen purification. The TP antibody-reactive antigens (TP-Ags) were isolated from both the ICWF-ASWS and the ID-TP reference antigen (75F+L) by purification procedures involving lectin affinity chromatography, electroelution from preparative SDS-polyacrylamide gels, gel filtration, and ion-exchange chromatography. Details of these purification steps are presented below.

(i) ConA affinity chromatography. The TP-Ags were concentrated by lectin affinity chromatography on a column (1.2 by 22 cm) containing ConA (Sigma) covalently linked to CNBr-activated Sepharose 4B (Sigma). The dried sample (10 mg) was dissolved in 0.02 M citric acid–Na_2HPO_4 buffer (pH 7.6) containing 0.5 M NaCl, 2.5×10^{-6} M $MnCl_2 \cdot 4H_2O$, and 9.0×10^{-6} M $CaCl_2$ and applied to the column which was equilibrated with buffer. Nonbound fractions were eluted at a descending flow rate of 40 ml/h and monitored at an A_{280}. After the A_{280} of the effluent returned to base line, bound components were eluted in the same buffer as described above but adjusted to pH 4.0 (16, 24). The eluate fractions showing the highest absorbance (A_{280}) were pooled. The effluent and eluate fractions were dialyzed separately against distilled water as described above, and the retained material was lyophilized. The effluent fractions were not further examined in this study. This method of desorption from the ConA affinity column by a reduction in the pH of the elution buffer was used rather than the conventional method of elution by competition with methyl α-D-mannopyranoside (16). We desorbed the ConA-bound fraction, using a reduced pH to avoid possible contamination of the sample with the competing sugar, which could have influenced results of subsequent carbohydrate analyses (6).

(ii) Electroelution of SDS-polyacrylamide gels. The pooled eluate fractions from the ConA affinity column were first separated by SDS-PAGE with a 7.5% separating gel. The lane containing the standards and a single adjacent lane to which the sample was added were cut from the gel and electrotransferred as described above. The Immobilon membrane-transferred standards were stained with Coomassie blue R-250 (Bio-Rad), while the transferred sample was reacted with ConA-peroxidase. The bands of interest were located in the unstained gel by carefully realigning them next to the lanes which contained the stained standards and ConA-blotted sample. This method was used to locate the higher-molecular-size TP-Ag (120 kDa) in the unstained, reducing gel separation of the ConA-bound, ICWF-ASWS fraction because of its weak staining with Coomassie blue R-250 and unsatisfactory resolution by the silver staining method. A narrow zone across the untreated gel adjacent to the band which was located by ConA reactivity was excised, and the contents were released from the gel by electroelution as previously reported (8). The lower-molecular-size TP-Ag (110 kDa) was easily located in reducing SDS-polyacrylamide gel separations of the ConA-bound fraction of the ID-TP reference antigen (75F+L) by Coomassie blue R-250 staining. The two isolated samples were separately electrodialyzed in an electroseparation system (Elutrap; Schleicher & Schuell Inc., Keene, N.H.). In this last step, the samples in electroelution running buffer, which contained 0.02% SDS (8), were each placed between two BT1 exclusion membranes (Schleicher & Schuell) and electrodialyzed against 100 mM ammonium acetate (pH 8.9) for 5 h at 100 V (constant voltage) to remove SDS. The retained material between the membranes was removed and lyophilized.

(iii) Gel filtration by HPLC. The dried, 120-kDa sample isolated by electroelution was resolubilized in filtered phosphate-buffered saline (PBS [0.05 M phosphate buffer containing 0.15 M NaCl]; pH 7.0), applied to a Superose 12HR 10/30 gel filtration (GF) column (Pharmacia Fine Chemicals, Piscataway, N.J.), and subjected to high-pressure liquid chromatography (HPLC) as previously described (8). A model HP1090 liquid chromatograph equipped with a diode array detector (Hewlett-Packard Co., Palo Alto, Calif.) was used which permitted peak purity analysis. High-molecular-

size GF standards (Sigma) were prepared in the same manner and separated by GF-HPLC under the same conditions as described above for the test sample. The standards included β-galactosidase (tetramer, 540 kDa; dimer, 268.5 kDa; monomer, 134.25 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa). β-Amylase was used as an external standard during each HPLC separation of the electroeluted sample. Estimates of the molecular size of the sample fractions were based on comparison of retention times (RT) of the sample and standards. Portions of sample fractions obtained by HPLC were tested directly in the ID-TP assay. Alternatively, pooled fractions were desalted and concentrated by centrifugation (700 × *g*, 5 min, 4°C) by using a Micropartition System MPS-1 (Amicon Corp., Danvers, Mass.) equipped with a type YMT ultrafiltration membrane (molecular size cutoff, 10 kDa; Amicon), washed four times with HPLC-grade water, and lyophilized.

(iv) Ion-exchange chromatography. The electroeluted 110-kDa sample was separated by ion-exchange chromatography with DEAE-Sephacel (bed volume, 14.7 ml; Pharmacia). The dried sample was redissolved in 0.02 M sodium phosphate buffer (pH 7.4) and eluted with a 0.01 to 0.5 M linear sodium chloride gradient at a flow rate of 0.2 ml/min. Fractions (0.95 ml) were monitored by determining the A_{280}, directly tested for reactivity in the ID-TP assay, or desalted and concentrated by ultrafiltration as described above.

2D-IEP and advancing line-IEP. The tandem two-dimensional immunoelectrophoresis (2D-IEP) procedure (3, 7) was used to identify antigenic components of the crude and ConA-bound fractions. Antigen identification was based on use of the CDN–anti-CDN reference system (5). The reference antigen (CDN) used in these gels was prepared as reported earlier (5). Other details of the tandem 2D-IEP procedure are the same as previously described (8). The advancing line-IEP procedure used in this study has been previously reported (3, 5). The IEP reference antigen (89F+L) used in the intermediate gel was diluted 1:128 in electrophoresis buffer to produce the two advancing lines identified as reference antigens 2 (Ag2) and CS (AgCS) (3). The position of these two advancing lines relative to the intermediate gel was reversed in comparison with that in our previous reports (3, 5). The anti-CDN immunoglobulin used in the present study was derived from a newly hyperimmunized burro (8), and the ratio of antibody titers to these two reference antigens has apparently changed. The advancing line-IEP procedure was used to examine the antigenic composition of the crude, ConA-bound, electroeluted, and DEAE-separated fractions of the ICWF-ASWS and ID-TP reference antigen (75F+L).

TP assay. The TP assay reported by Smith and co-workers (28) was performed. Because of the uncertainty of whether the ID-TP and TP assays detect the same precipitinogen (9), we examined reactivity of the chromatographically purified TP-Ags with sera from patients with coccidioidomycosis in both tests. Five patient serum samples obtained from Theo N. Kirkland (Veterans Administration Medical Center, San Diego, Calif.) were determined to have ID-TP titers of 1:2 to 1:8. All sera from patients with coccidioidomycosis used in this study were also tested in the ID assay for the presence of CF antibodies (17) and all were shown to be ID-CF positive. Portions (100 μl) of each undiluted serum sample were pooled and transferred to a 3-ml, acid-cleaned glass tube, and 0.005% thimerosal was added to prevent microbial activity during incubation. The reactivities of the two chromatographically isolated fractions were first tested separately by using a portion (50 μl) of the pooled sera in the ID-TP assay. Portions (200 μl) of the remaining pooled patient sera were overlaid with 20 μg of each chromatographic fraction in 200 μl of PBS (pH 7.4) and incubated in acid-cleaned glass tubes for 24 h at 37°C. The cohesiveness of the buttonlike precipitin was tested by sharply flicking the bottom of the tube (28). Portions of five control serum samples (undiluted) from patients admitted to the hospital with no systemic or pulmonary mycoses (provided by Athis da Silva, Brackenridge Hospital, Austin, Tex.) were prepared and tested as described above. All control sera were examined in the ID assays and shown to be ID-TP and ID-CF negative.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was conducted with an indirect screening kit (Kirkegaard & Perry) by the procedure described previously (4). The five ID-TP-positive and five control serum samples cited above were used. Each serum sample was tested separately in the ELISA for binding of IgM and IgG to the chromatographically isolated antigens by using goat anti-human IgM or goat anti-human IgG, respectively, each of which was conjugated to peroxidase (Kirkegaard & Perry). Optimal antigen concentration was determined by block titration (4). An antigen concentration of 124 ng/ml of buffer and a 1:1,500 dilution of test sera (control and patient serum samples) in blocking solution were chosen for comparative purposes. This serum dilution was chosen because it fell at the midpoint of the optimal dilution range of the test sera which were titrated in the ELISA. Assays with sera in the absence of antigen and with antigen in the absence of sera served as controls. All sera were tested in triplicate wells.

RESULTS

Reactivities of crude antigenic fractions in ID-TP assay. ID assays of ICWF-ASWS preparations revealed two precipitin bands formed by reaction with the human reference antibody after incubation at room temperature for 72 h. A sharp band closest to the sample well could be distinguished from an adjacent diffuse band when the antigen concentration in the well was 1 μg in 30 μl of PBS (arrowheads in Fig. 1A). Both the ID-TP reference antigen (75F+L) used in this assay and the ICWF-ASWS preparation were heated to 60°C for 30 min in PBS prior to their analysis in the ID-TP system. The same results were obtained with the Meridian ID-TP system (data not shown).

SDS-PAGE separation and ConA reactivity. The protein components of ICWF-ASWS were not well defined in SDS-polyacrylamide gels by either the silver or Coomassie blue R-250 staining procedures. Three polypeptide bands were visible with Coomassie blue R-250 staining in 7.5% gels under reducing conditions. The components had estimated molecular sizes of 66, 58, and 52 kDa (Fig. 1B). Other bands were obscured by a smear of stain which increased in intensity toward the running front of the gel and was most intense in the silver-stained gels. When components of this same gel separation of ICWF-ASWS were electrotransferred to an Immobilon membrane, which was then incubated with ConA-peroxidase and exposed to developing solution, a single, intense 120-kDa band was visible (Fig. 1B). The concentrated, ConA-bound (eluate) fraction of ICWF-ASWS obtained by lectin affinity chromatography (1 mg) was examined by SDS-PAGE under reducing conditions in both 7.5 and 14% gels (Fig. 1B). A single 120-kDa band, which was weakly stained by Coomassie blue R-250, was visible in the 7.5% gel. We previously estimated the molec-

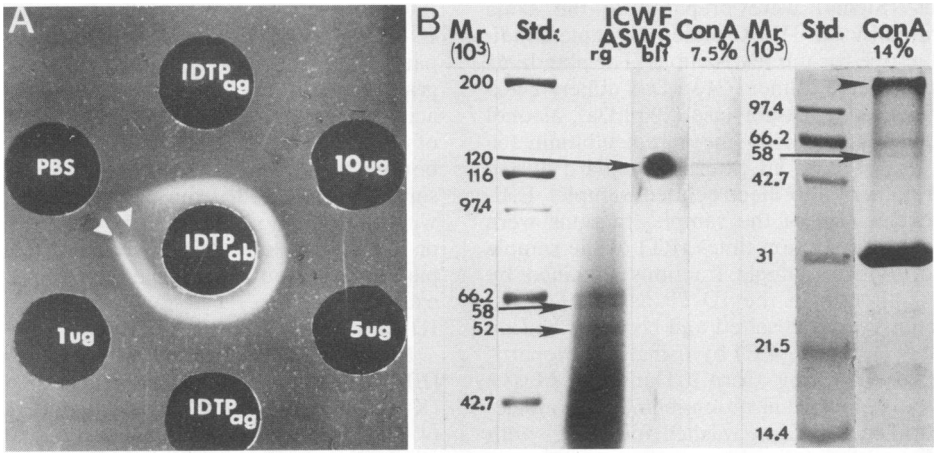

FIG. 1. Immunoreactivity (A) and composition (B) of ICWF-ASWS. (A) Wells of the ID plate contain human reference antibody (ab) and the TP reference antigen (ag). The concentrations of the ICWF-ASWS in test wells were 10, 5, and 1 μg in 30 μl of PBS. Arrowheads in panel A indicate two apparently separate precipitin bands. (B) Reducing SDS-polyacrylamide gel (rg) separation and ConA-peroxidase blot (blt) of the ICWF-ASWS were conducted by using a 7.5% slab gel. The bound (eluate) fraction from the ConA chromatographic column revealed a single 120-kDa component in the 7.5% gel (ConA, 7.5%) and several components (31 to 66 kDa) in the 14% gel (ConA, 14%). The latter gel also showed Coomassie blue R-250-stained material at the interface of stacking and separating gels (arrowhead). Relative molecular mass (M_r) of samples and standards (Std.) are shown.

ular size of this band in error as 124 kDa (D. Kruse and G. T. Cole, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, F34, p. 463). The major polypeptide bands revealed in the 14% gel separation of the ConA-bound fraction included 66- and 31-kDa components, as well as Coomassie blue R-250-stained material at the interface of the stacking and separating gels (arrowhead in Fig. 1B). The latter material was apparently excluded from the 14% gel during electrophoresis.

Reactivities of electroeluted fractions in ID-TP assay. The 120-kDa fraction electroeluted from 7.5% SDS-polyacrylamide gel separations of the ConA-bound fraction of ICWF-ASWS was tested for ID-TP activity against the reference serum and against two additional TP-positive sera from patients with coccidioidomycosis (Fig. 2A). The prominent precipitin band between the two wells containing the reference antigen and antibody was fused with the precipitin band formed between wells containing the electroeluted sample and the reference antibody. Fusions of precipitin bands also occurred between the wells containing the ID-TP-positive patient sera (serum sample accession nos. 4 and 7), the electroeluted sample, and the reference system. The SDS-PAGE-isolated fraction demonstrated a similar reactivity in the Meridian ID-TP assay (data not shown).

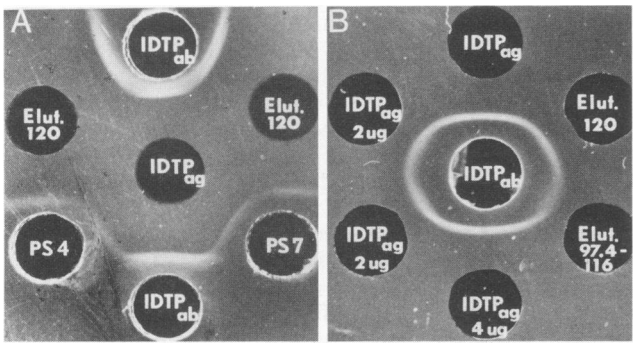

FIG. 2. (A) Reactivity of electroeluted 120 kDa fraction (Elut. 120) isolated from 7.5% SDS-polyacrylamide gel separation of the ConA-bound fraction of ICWF-ASWS, with human reference antibody (ab) and TP reference antigen (ag), as well as sera from patients with coccidioidomycosis (PS; accession nos. 4 and 7) in the ID-TP assay. (B) Reactivity of electroeluted 120-kDa fraction (Elut. 120) and electroeluted 97.4- to 116-kDa fraction (Elut. 97.4–116) from 7.5% SDS-polyacrylamide gel separation of the crude ID-TP reference antigen (75F+L) with human reference antibody (ab) and TP reference antigen (ag) in the ID-TP assay. The concentrations of reference antigen in test wells were 2 or 4 μg in 30 μl of PBS.

SDS-PAGE separations (7.5% gels) of the ID-TP reference antigen (75F+L) revealed a 120-kDa band that was weakly stained with Coomassie blue R-250 and a well-stained band at approximately 110 kDa (data not shown). We excised and electroeluted the 120-kDa band as well as the region of SDS-polyacrylamide gel (7.5%) separation of the ID-TP reference antigen between the 97.4- and 116-kDa standards. These fractions were tested separately in the ID-TP assay. The results of a representative test of the reactivity of the 97.4- to 116-kDa fraction is shown in Fig. 2B. Fusions of the precipitin bands between the human reference antibody and the electroeluted 120-kDa band, the electroeluted 97.4- to 116-kDa band, and the ID-TP reference antigen are clearly visible.

Antigenic composition of isolated fractions in 2D-IEP and advancing line-IEP gels. The anodal well of the 2D-IEP plate in Fig. 3A contained the crude ICWF-ASWS, while the cathodal well contained CDN. A prominent tandem peak was visible which was identified as Ag2 on the basis of comparison of the pattern of precipitin peaks with that of the control gel of the reference antigen (4, 5) (data not shown). Other tandem peaks are also visible in this gel (Fig. 3A, arrows). The crude ID-TP reference antigen (75F+L) revealed prominent AgCS and Ag2 tandems when examined under these same conditions (3) (data not shown). In Fig. 3B, the sample in the anodal well was the ConA-bound fraction obtained by lectin affinity chromatographic separation of the ICWF-ASWS. Some of the same tandems with reference precipitins that are visible in this gel were also revealed in

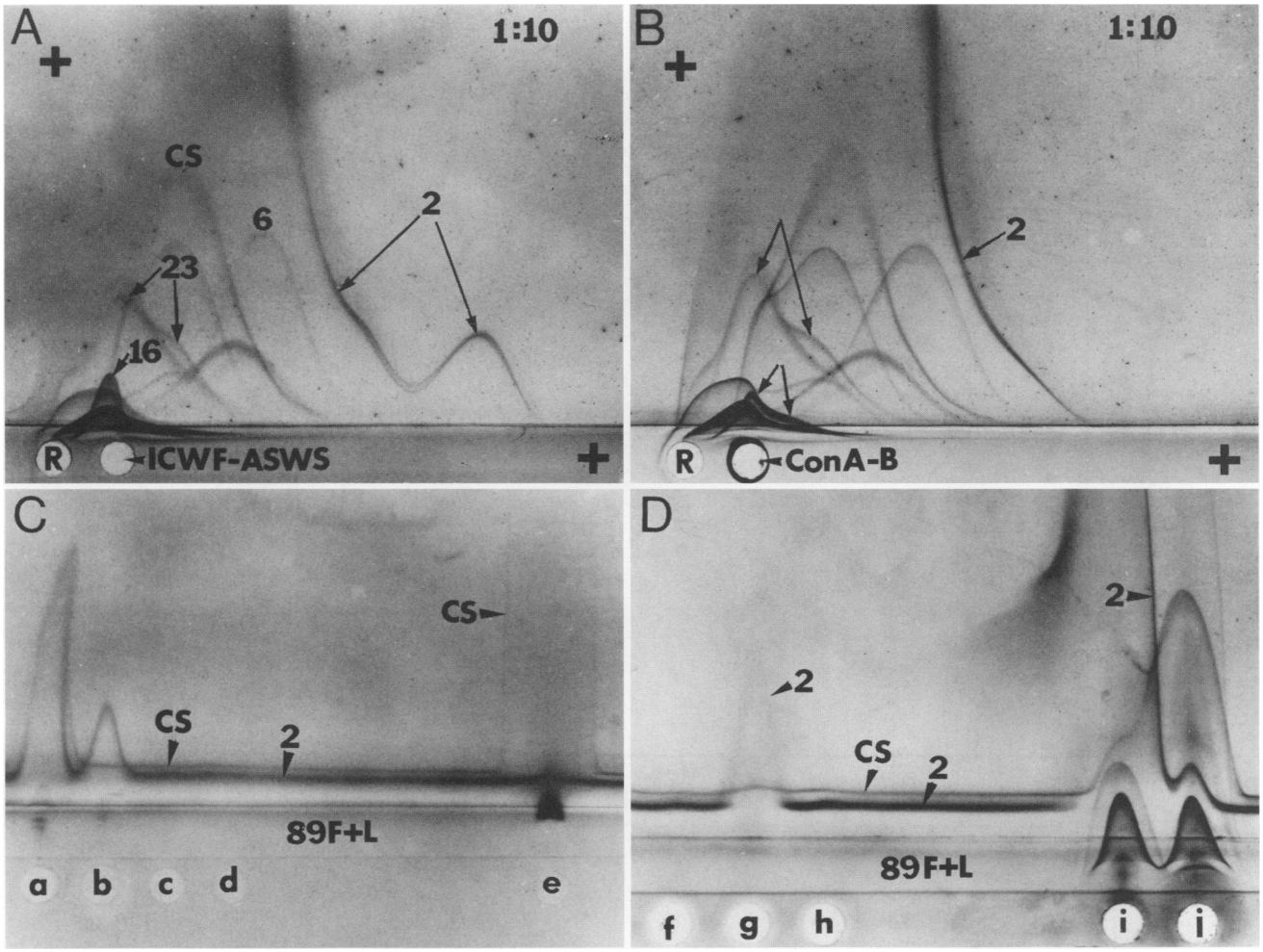

FIG. 3. Tandem 2D-IEP (A and B) and advancing line-IEP gels (C and D) of isolated fractions showing antigenic composition. The cathodal wells of the tandem 2D-IEP gels contained coccidioidin (R; 50 μg in 20 μl of PBS), while the anodal wells contained 200 μg in 20 μl of either the ICWF-ASWS (A) or the ConA-bound (ConA-B) fraction of the ICWF-ASWS (B). The upper gels contained burro anti-CDN immunoglobulin diluted 1:10 in electrophoresis buffer. The plus signs indicate the anodes and direction of migration in each dimension. Precipitin peaks are labeled according to the CDN–anti-CDN reference system (5, 7). Tandem peaks are indicated by paired arrows. The following test wells in advancing line-IEP gels (C and D) contained the indicated fractions, each of which was solubilized in 20 μl of PBS: a, ID-TP reference antigen (75 F+L; 5 μg); b, ICWF-ASWS (5 μg); c, ConA-bound (eluate) fraction of ICWF-ASWS (5 μg); d, electroeluted 120-kDa fraction from SDS-PAGE separation of ICWF-ASWS (5 μg); e, acetone precipitate of the soluble conidial wall fraction (5) used as an AgCS reference fraction (1 μg); f, electroeluted 120-kDa fraction from SDS-PAGE separation of reference antigen (75 F+L) (5 μg); g, electroeluted 97.4- to 116-kDa fraction from SDS-PAGE separation of ICWF-ASWS (1 μg); h, electroeluted 66-kDa fraction from SDS-PAGE separation of ICWF-ASWS (1 μg); i, conidial cytosol fraction (10 μg); j, soluble conidial wall fraction (5) (10 μg). The upper gels contained burro anti-CDN immunoglobulin diluted 1:7 in electrophoresis buffer. The intermediate gel contained the reference antigen (89F+L) diluted 1:128 in electrophoresis buffer. The method of identification of the two advancing lines as Ag2 and AgCS, which was based on correlation to the reference system, has been previously reported (3).

Fig. 3A. However, no tandem with the reference Ag2 peak was detected. In contrast, the ConA-bound fraction of the ID-TP reference antigen (75F+L) showed a prominent tandem with Ag2 (data not shown). We further examined the antigenic composition of isolated fractions by advancing line-IEP (Fig. 3C and D). The two reference precipitin lines were identified as Ag2 and AgCS by comparison with the established reference system (3). In Fig. 3C, the two crude samples used in this study as sources of the TP-Ags (i.e., ICWF-ASWS and ID-TP reference antigen 75F+L) were shown to contain Ag2. As stated above, the ID-TP reference antigen also contained AgCS (Fig. 3C). However, the ConA-bound fraction of the ICWF-ASWS and both the electroeluted 120- and 66-kDa fractions obtained from SDS-polyacrylamide gel separations of this lectin-bound sample lacked Ag2 and AgCS (Fig. 3C and D). The 31-kDa fraction of the ConA-bound sample (Fig. 1B) also showed no fusion with the Ag2 and AgCS precipitin lines (data not shown). On the other hand, the electroeluted, ID-TP-reactive fraction obtained from the 97.4- to 116-kDa region of SDS-polyacrylamide gel separations of the crude ID-TP reference antigen (75F+L), crude ICWF-ASWS fraction, or ConA-bound fraction of the ID-TP reference antigen was shown to contain Ag2 in the advancing line-IEP gel (Fig. 3D).

GF-HPLC separation of 120-kDa fraction. Figure 4A shows a representative chromatogram of a GF-HPLC separation of the electroeluted, 120-kDa fraction obtained from SDS-polyacrylamide gels of the ConA-bound components of the

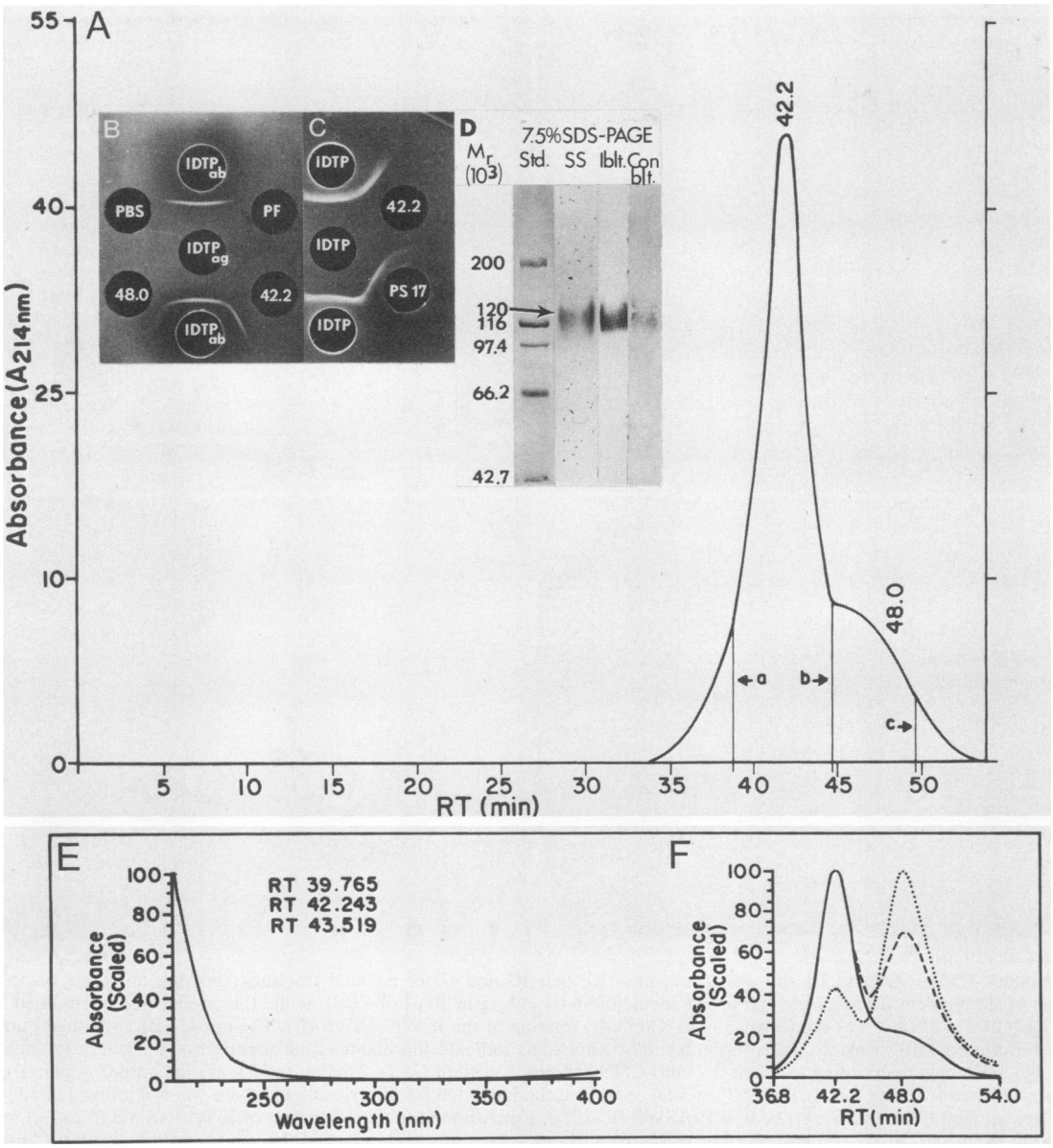

FIG. 4. Representative elution profile of GF-HPLC separation of electroeluted 120-kDa fraction from 7.5% SDS-polyacrylamide gel separation of ICWF-ASWS and ID-TP reference antigen (75 F+L) (A). Major and minor peaks had RTs of 42.2 and 48.0 min, respectively. Portions (30 μl) of pooled HPLC fractions were tested for reactivity in ID-TP assays (B and C) with human reference antibody (ab), TP antigen (ag), and serum from a patient with coccidioidomycosis (patient serum accession no. 17). Test wells contained PBS or portions (30 μl) of pooled fractions with RTs of 10.0 to 37.9 min (pooled fraction [PF]), a to b (42.2-min peak), or b to c (48.0-min peak). (D) Results of testing homogeneity of the major peak (42.2 min) obtained by GF-HPLC by using silver-stained, reducing SDS-polyacrylamide gel (7.5%) separation (SS), immunoblot analysis with ID-TP-positive patient serum (Iblt.), and ConA blotting (Con blt.). Estimated M_rs of samples and standards (Std.) are shown. (E) Absorption spectrum for the major fraction in panel A shown as a composite of the spectrum (A_{210} to A_{400}) of eluted sample with RTs of 39.8, 42.2, and 43.5 min. (F) Combined elution profiles for three absorption wavelengths. Symbols: ——, A_{214}; – – – –, A_{254}; ....., A_{280}.

ICWF-ASWS. The elution profile was highly reproducible for separate preparations of the electroeluted fraction. The RT for absorbance peaks (A_{214}) of the major and minor components were 42.2 and 48.0 min, respectively. In addition to collection of these two peaks (range of RT, 38.0 to 44.5 min and 44.6 to 49.5 min, respectively), material which may have eluted prior to the appearance of the major peak (range of RT, 10.0 to 37.9 min) but which showed no absorbance (A_{214}) was pooled. The pooled fraction was concentrated to approximately 30 μl by using the Amicon MPS-1 system. This concentrated, pooled fraction and 30 μl of the eluted major and minor peaks obtained directly by high-pressure liquid chromatography without subsequent sample concentration (42.2 and 48.0 min, respectively) were separately added to wells of the ID plate (Fig. 4B). Only the latter two peaks showed ID-TP assay reactivity. A distinct line of identity was established between the sample representing the major peak and the ID-TP reference system. This same sample was used in ID-TP assays with sera from patients with coccidioidomycosis previously shown by ID to

be TP positive, as well as sera from control patients. The results of a representative ID-TP assay of the reactivity between a TP-positive serum sample (serum sample accession no. 17) and the HPLC fraction which eluted at 42.2 min are shown in Fig. 4C. Lines of identity were shown between the ID-TP reference system, the TP-positive patient serum sample, and the HPLC fraction. The fractions corresponding to the major and minor peaks of the HPLC separation were also reactive in the Meridian ID-TP assay (data not shown). All control sera lacked reactivity with the chromatographic fractions in the ID-TP assay.

The 120-kDa band electroeluted from SDS-polyacrylamide gel separations of the ConA-bound fraction of the ID-TP reference antigen (75F+L) was also subjected to GF-HPLC fractionation. The same elution profile was revealed as in Fig. 4A, and the same two fractions were reactive in the ID-TP assay (data not shown).

Representative silver-stained, SDS-polyacrylamide reducing gel (7.5%) separation of the major peak (RT, 38.0 to 44.5 min), an immunoblot with an ID-TP-positive serum sample from a patient, and a ConA blot of this same gel separation are shown in Fig. 4D. A single, although diffuse band with a molecular size of approximately 120 kDa was visible in each case.

A composite spectrum of the major peak generated by absorbance of the sample over a range of wavelengths from 210 to 400 nm is shown in Fig. 4E. Spectral data for this range were obtained at each RT indicated. The appearance of coincident spectra is evidence of a single compound. A composite, scaled chromatogram obtained by simultaneous absorbance of the eluate using wavelengths of 214, 254, and 280 nm is presented in Fig. 4F. While the first major peak showed greatest absorbance at 214 and 254 nm, the minor peak revealed an absorbance maximum at 280 nm. The retention times of the major and minor peaks determined by absorbance at the three different wavelengths were identical (i.e., 42.2 and 48.0, respectively) (Fig. 4F).

Estimates of the molecular sizes of the two gel filtration components of the electroeluted 120-kDa TP-Ag were based on comparison with the elution profiles of GF calibration standards. β-Galactosidase (grade VIII from *Escherichia coli*; Sigma) eluted as a tetramer (540 kDa), dimer (268.5 kDa), and monomer (134.25 kDa) (12). The retention times of the GF standards were plotted against the molecular size and showed a predicted, linear relationship. The molecular size of the major and minor ID-TP-reactive fractions isolated by GF-HPLC were extrapolated as 240 and 120 kDa, respectively.

DEAE separation of 110-kDa fraction. The ConA blot of a reducing gel (7.5%) separation of the ID-TP reference antigen (75F+L) in Fig. 5 showed both 120- and 110-kDa bands. In contrast, only the 120-kDa component was revealed under the same conditions in gel separations of the ICWF-ASWS (cf. Fig. 1B). Different concentrations of sample (0.25 to 1.0 mg) were applied to three lanes of the SDS-polyacrylamide gel and lectin blotted (Fig. 5). The results suggest a slightly higher affinity of the 120-kDa fraction than of the 110-kDa fraction for ConA. When the gel separations of the same titration of ConA-bound ID-TP reference antigen were immunoblotted with TP-positive serum from patients, a broad smear was revealed above and slightly below the 120-kDa band, whereas a distinct band was shown at 110 kDa. Because of the possibility of coelution of the 110-kDa fraction and other immunoreactive components from this gel, DEAE separation of the electroeluted 110-kDa band was performed. A representative DEAE elution profile is shown

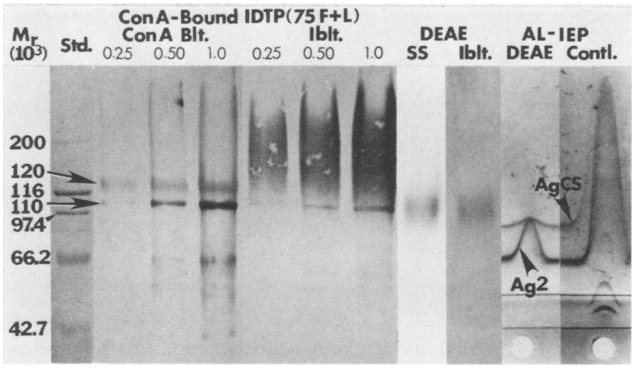

FIG. 5. SDS-polyacrylamide gel (7.5%) separations of ConA-bound fraction of ID-TP reference antigen (75F+L) which were subjected to ConA blotting (ConA Blt.) and immunoblotting (Iblt.). The latter was performed by using the pool of ID-TP-positive patient sera. The amount of sample added to each lane was 0.25, 0.50, or 1.0 mg. The immunoreactive fraction eluted from the DEAE column with 0.20 to 0.25 M NaCl is shown in a silver-stained (SS) SDS-polyacrylamide gel (7.5%) and an immunoblot (Iblt.) of this gel separation by using a pool of ID-TP-positive patient sera. The antigenic composition of the same DEAE fraction was examined in a CDN–anti-CDN reference system by advancing line-IEP (AL-IEP). The DEAE sample (12.5 μg in 20 μl of PBS) was added to the left well, and reference antigen (Contl.; 89F+L diluted 1:8 in PBS) was added to the right well. Intermediate gel contains 89F+L diluted 1:128 in electrophoresis buffer. Upper gel contains anti-CDN immunoglobulin diluted 1:7 in electrophoresis buffer. Advancing lines are identified as Ag2 and AgCS. Std., Standards.

in Fig. 6. Two peaks were revealed when fractions were monitored at an A_{280}. The first fraction was eluted early from the DEAE column with 0.02 M phosphate buffer, while the second fraction was eluted after initiation of the NaCl gradient (approx. 0.2 to 0.25 M NaCl). Pooled fractions of each peak were reactive in the ID-TP assay. These same fractions were separately concentrated and examined by SDS-PAGE, immunoblot analysis, and advancing line-IEP. A single 110-kDa band was revealed in the silver-stained gel

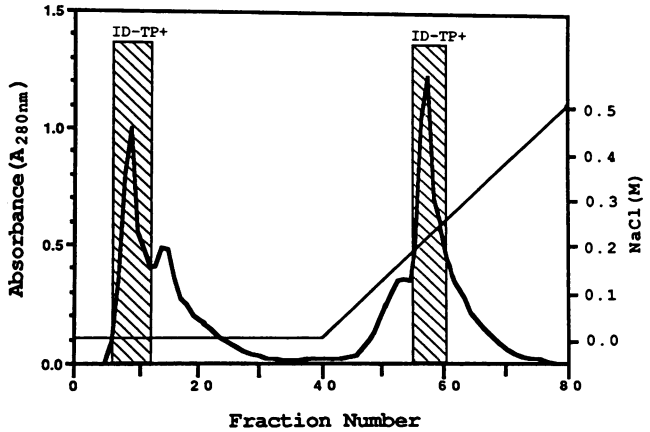

FIG. 6. Ion-exchange (DEAE) separation of the 110-kDa electroeluted sample obtained from reducing SDS-polyacrylamide gel (7.5%) separation of the ConA-bound fraction of the ID-TP reference antigen (75F+L). Pooled fractions that were reactive in the ID-TP assay (ID-TP$^+$) were examined in silver-stained SDS-polyacrylamide gels (7.5 and 14.0%). Only the second reactive peak (fractions 55–60) produced a visible band (110 kDa) in the 7.5% SDS-polyacrylamide gel (cf. Fig. 5).

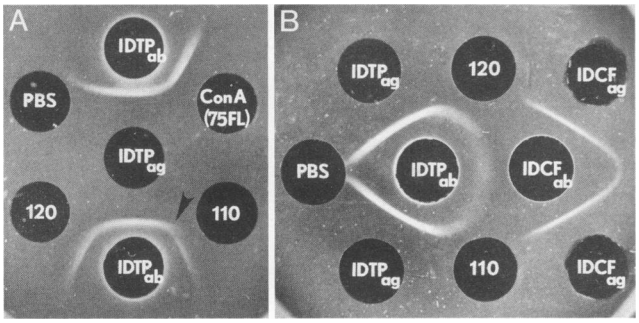

FIG. 7. (A) Reactivity of ConA-bound fraction of ID-TP reference antigen (ConA [75F+L]), DEAE-isolated 110-kDa fraction, and GF-HPLC-isolated 120-kDa fraction with human reference antibody and TP reference antigen. The arrowhead indicates the spur at the region of fusion of reference precipitin and 110-kDa precipitin bands. (B) Reactivity of GF-HPLC-isolated 120-kDa fraction and DEAE-isolated 110-kDa fraction in both ID-TP and ID-CF reference systems. Abbreviations: ab, antibody; ag, antigen; 120, 120-kDa fraction; 110, 110-kDa fraction.

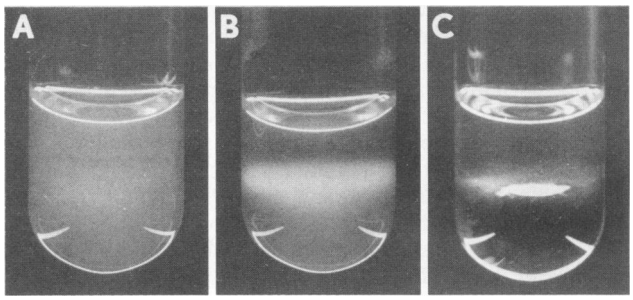

FIG. 8. Results of representative TP assay in which the chromatographically isolated 120-kDa TP-Ag was added to pooled control sera (A) or pooled sera from ID-TP-positive patients with coccidioidomycosis and incubated at 37°C for 2 h (B) and 24 h (C).

of the bound peak (fractions 55 to 60; Fig. 5), while the concentrated, unbound peak (fractions 6 to 12) showed no gel bands (data not shown). A single 110-kDa band was also shown on Immobilon membranes when the electrophoresis gel which separated the DEAE-bound peak was immunoblotted with ID-TP-positive serum from patients (Fig. 5). The advancing line-IEP gel in Fig. 5 demonstrated that the DEAE-bound peak contained Ag2. The slight deflection of the AgCS precipitin line was considered to be an artifact and may have been caused by residual buffer in the DEAE-bound sample. Silver-stained reducing SDS-PAGE (14%) separation of the DEAE-bound sample showed no band at 19 kDa (8).

Reactivity of 120- and 110-kDa fractions in ID-TP, ID-CF, and TP assays. The ID plates shown in Fig. 7A and B included both the ID-TP and ID-CF reference systems. The test wells contained the ConA-bound fraction of the ID-TP reference antigen (75F+L), the 120-kDa fraction isolated by GF-HPLC, and the 110-kDa fraction isolated by DEAE chromatography. The ConA-bound and isolated TP-Ags formed precipitin bands which fused with the ID-TP reference precipitin. A spur was visible between the fusion of the 110-kDa band and that of the ID-TP reference system in Fig. 7A (arrowhead). No fusion occurred between the ID-CF reference precipitin and the precipitins of the test samples (Fig. 7B).

The reactivities of the two chromatographically isolated fractions with patient antibody was also examined in the classical TP test. A precipitin ring was visible in the test tube within 2 h after adding the solution which contained the GF-HPLC-isolated, 120-kDa fraction to the pooled, ID-TP-positive patient sera (Fig. 8B). No such precipitin ring was visible in the tube containing the 120-kDa fraction plus the pooled control sera (Fig. 8A). The ring which formed at the interface of the two solutions was dispersed by mixing the contents of the tube. After incubation at 37°C for 24 h, a buttonlike precipitin formed (Fig. 8C) which could not be disrupted by sharply flicking the bottom of the test tube. A precipitin band also formed between wells of the ID plate which contained these same pooled sera and the 120-kDa antigen preparation. The precipitin band fused with that of the ID-TP reference system (data not shown). An identical TP assay was conducted by using the chromatographically purified 110-kDa fraction, and the same results were obtained.

ELISA. Both the chromatographically purified 120- and 110-kDa fractions showed higher patient IgM antibody binding than did patient IgG antibody binding in the ELISA. The average absorbance values (A_{414}) of the five test sera after reaction with the 120-kDa fraction by using the specific anti-human IgM and IgG conjugates were 1.125 ± 0.016 and 0.406 ± 0.016, respectively. The corresponding absorbance values for IgM and IgG binding to the 110-kDa fraction were 0.702 ± 0.012 and 0.118 ± 0.012, respectively.

DISCUSSION

Reactivity of the crude alkali extract of the inner conidial wall or the crude mycelial culture filtrate plus toluene lysate of *C. immitis* with TP-positive sera from patients with coccidioidomycosis in the standardized ID assay revealed two precipitin bands. In this report we presented evidence for the purification of 120- and 110-kDa components of these two crude antigenic fractions which showed reactivity with sera from patients with coccidioidomycosis in both the classical TP assay and the ID-TP assay. On the basis of our results of examinations of these isolated fractions in the ELISA, we suggest that formation of the diagnostic precipitin in the ID-TP assay is the product of mainly patient IgM antibody binding to both 120- and 110-kDa components of the reference antigens.

Previous investigators have reported isolation of TP antibody-reactive fractions which have several characteristics in common with the purified TP-Ags described in this report. The incomplete precipitating antigen, which Cox and Britt (9) identified in 2D-IEP gels as a component of their ASWS cell wall extracts (9–11), coccidioidin, and spherulin (10), was shown to be reactive with IgM precipitin antibody. Although the incomplete precipitating antigen was demonstrated to be a single precipitinogen by 2D-IEP, which suggests antigenic homogeneity, the presence in the same isolated fraction "of antigens which do not precipitate with antibody or components that are not antigenic cannot be excluded. . ." (9). The ID-TP antigen was isolated by using a combination of immunoaffinity and ion-exchange chromatographies and was shown to be resistant to heat and pronase digestion but susceptible to periodate oxidation (9). In a later report (13), this same antigen isolated from CDN was used to identify reactive hybridomas derived from spleen cells of spherulin-immunized BALB/c mice. A monoclonal antibody detected a diffusely staining band with a molecular size distribution of 130 to 330 kDa in immunoblots of reducing

SDS-polyacrylamide gel separations of the ID-TP antigen. The same monoclonal antibody also weakly cross-reacted with an epitope of another antigen, which was estimated to have a molecular size of 110 kDa and was suggested to be Ag2 (11, 13). When the 120-kDa TP-Ag described in this report was examined by tandem 2D-IEP or advancing line-IEP, no fusion with precipitins of the reference antigen were visible. The 120-kDa TP-Ag, therefore, was not detected in the CDN–anti-CDN reference system, at least when our previously reported methods of IEP were used (7, 8). On the other hand, the 110-kDa TP-Ag was identified as Ag2 by advancing line-IEP by using this same reference system. The 110-kDa fraction was isolated by electroelution of SDS-polyacrylamide gel separations of both the crude ICWF-ASWS and the ConA-bound fractions of the ID-TP reference antigen (75F+L). It is possible that a common epitope(s) exists between the 120-kDa fraction and Ag2, perhaps associated with the polysaccharide structure of these two electrophoretically distinct fractions.

Zimmer and Pappagianis (32) reported isolation of a fraction that was responsible for production of the antigen-antibody precipitate in the TP test. They used pronase digestion followed by gel filtration and ion-exchange chromatography to separate the TP-Ag from the precipitin-antibody complex. The fractions with ID-TP reactivity which were detected after DEAE chromatography had molecular sizes of 225 and 140 kDa, based on gel filtration estimates. The authors suggested that these two fractions are components of a single antigen producing the ID-TP reaction. Exhaustive digestion of the crude fraction with pronase (72 h, 37°C) may have resulted in some alteration in composition of the 120-kDa TP-Ag reported here and total or partial loss of the 110-kDa TP-Ag.

In another study which reported identification of the TP-Ag, Calhoun and co-workers (1) examined immunoblot reactivity of components of the mycelial culture filtrate plus toluene lysate and spherule autolysate of *C. immitis* by using sera from both patients with coccidioidomycosis and immunized rabbits. They suggested that a fraction of approximately 100 kDa was the TP-Ag. Fractions containing the 100-kDa-reactive band, however, also included diffuse components of higher molecular size, as revealed in a subsequent investigation (15). In that study (15), which involved gel filtration of a toluene-extracted spherule lysate of the Silveira strain of *C. immitis*, pooled fractions showing the highest TP reactivity (i.e., fractions 49 to 56) included components with molecular sizes of approximately 40 to 300 kDa. TP activity of the toluene-extracted spherule lysate was shown to persist even after autoclaving at 120°C.

Our estimates of 120 and 110 kDa for the molecular sizes of the TP-Ags from SDS-polyacrylamide gels were determined under reducing conditions in a 7.5% gel. The precipitin antibody-reactive fractions of both the ICWF-ASWS and the ID-TP reference antigen were excluded from the 14% gel. Our use of 7.5% denaturing SDS-polyacrylamide gel separations of the crude immunoreactive starting material as an early step in the isolation of the TP-Ag excluded the antigen previously reported to be reactive with CF antibodies from patients with coccidioidomycosis. This antigen was identified as a 48-kDa band by SDS-PAGE under reducing conditions (31). The 120- and 110-kDa fractions formed precipitin bands in ID plates which were clearly separated from the ID-CF reference precipitin, but showed fusion with the ID-TP reference precipitin.

Gel filtration separation of the 120-kDa fraction revealed a major and minor component with estimated molecular sizes of 240 and 120 kDa, respectively. These estimates were based on precise reference to GF calibration standards and were reproducible for each sample preparation. In a subsequent study (6), analysis of the amino acid composition of the 120-kDa TP-Ag revealed cysteine, which may be associated with the formation of intermolecular disulfide bonds. One possibility is that the reduced and electroeluted 120-kDa TP-Ag undergoes dimerization when incubated in PBS as a result of reassociation of disulfide bonds. It is also possible that the larger, GF-HPLC-isolated fraction is simply the product of aggregation caused by self-association of the SDS-polyacrylamide gel-isolated fraction. However, both the 120- and 240-kDa GF fractions were reactive in the ID-TP assay. The latter showed the highest reactivity in this assay.

The major peak (240 kDa) obtained by GF-HPLC was examined for homogeneity in silver-stained, SDS-polyacrylamide reducing gels. A single band with an estimated molecular size of 120 kDa was observed. ConA blot analysis of this gel separation also revealed a single band with the same molecular size. The affinity of the 120-kDa TP-Ag for ConA, based on results of the lectin blots, suggests specific interactions of mannosyl residues, glucosyl residues, or both with the lectin (24). In a separate report (6), we have presented evidence that the 120-kDa antigen is a glycoprotein containing methylated mannose residues which bind patient antibody. Immunoblot analysis of SDS-polyacrylamide gel separations of the 120-kDa TP-Ag isolated from the ICWF-ASWS and ID-TP reference antigens revealed broad bands of immunoreactivity which appeared to extend into the region of the 110-kDa band. We suggest that immunoreactive residues of the 120-kDa TP-Ag dissociate from the glycoprotein during electrophoresis in the reducing gel, which could be partly responsible for the broad, reactive bands shown in the immunoblots.

Because of the possibility that such dissociated, immunoreactive residues may have coeluted with the 110-kDa fraction from SDS-polyacrylamide reducing gels of the ID-TP reference antigen (75F+L), the 110-kDa fraction was further separated by ion-exchange chromatography. The first DEAE peak, which was reactive in the ID-TP assay, was probably composed of carbohydrate residues. The material in this peak eluted from the column in 0.02 M phosphate buffer. Silver-stained SDS-polyacrylamide reducing gel separations of this concentrated fraction, however, failed to reveal any bands. The second ID-TP reactive DEAE peak was eluted with 0.2 to 0.25 M NaCl, and the concentrated fraction was revealed as a 110-kDa band in the silver-stained SDS-polyacrylamide gel and as Ag2 in the CDN–anti-CDN reference system. The immunoblot of the gel separation of this DEAE fraction also showed a 110-kDa band, which provides additional evidence that the 110-kDa TP-Ag was separated from the 120-kDa TP-Ag.

Results of lectin blot analyses of SDS-PAGE separations of the ID-TP reference antigen suggested that the 120-kDa TP-Ag has a slightly greater affinity for ConA than the 110-kDa TP-Ag does. This difference may account for the apparent absence of the 110-kDa component (and Ag2) in the ConA-bound fraction of the ICWF-ASWS sample. Another possible explanation, however, is that alkali extraction affected ConA binding of the 110-kDa fraction while it did not appreciably influence that of the 120-kDa fraction. Alkali-extracted wall fractions of *C. immitis* have been previously shown to be highly reactive with TP antibody in sera from patients with coccidioidomycosis (11). This study initially focused on the ICWF-ASWS fraction, which proved to be a

good source of the 120-kDa TP-Ag. This same TP-Ag was isolated in this study from the mycelial culture filtrate plus toluene lysate (ID-TP reference antigen) and showed a similar immunoreactivity. For the reasons indicated above, the latter was also used for isolation of the 110-kDa TP-Ag by ConA affinity chromatography.

Analysis of the chemical composition of the TP-Ags is necessary to obtain a better understanding of the nature of their reactivities with patient antibody. We have examined the composition and serologic reactivity of the 120-kDa TP-Ag (6). Results of comparable investigations of the 110-kDa TP-Ag will be reported in a future report.

ACKNOWLEDGMENTS

We are grateful for the technical assistance of S. Zhu, J. W. Chinn, K. R. Seshan, and J. Dominguez.

This investigation was supported by Public Health Service grant AI19149 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Calhoun, D. L., E. O. Osir, K. O. Dugger, J. N. Galgiani, and J. H. Law.** 1986. Humoral antibody responses to specific antigens of *Coccidioides immitis*. J. Infect. Dis. **154**:265–272.
2. **Catanzaro, A., and F. Flatauer.** 1983. Detection of serum antibodies in coccidioidomycosis by solid-phase radioimmunoassay. J. Infect. Dis. **147**:32–39.
3. **Cole, G. T., J. W. Chinn, L. M. Pope, and P. Starr.** 1985. Characterization and distribution of 3-*O*-methylmannose in *Coccidioides immitis*, p. 130–145. *In* H. Einstein and A. Catanzaro (ed.), Coccidioidomycosis. Proceedings of the Fourth International Conference on Coccidioidomycosis. National Foundation for Infectious Diseases, Washington, D.C.
4. **Cole, G. T., T. N. Kirkland, M. Franco, S. Zhu, L. Yuan, S. H. Sun, and V. N. Hearn.** 1988. Immunoreactivity of a surface wall fraction produced by spherules of *Coccidioides immitis*. Infect. Immun. **56**:2695–2701.
5. **Cole, G. T., T. N. Kirkland, and S. H. Sun.** 1987. An immunoreactive, water-soluble conidial wall fraction of *Coccidioides immitis*. Infect. Immun. **55**:657–667.
6. **Cole, G. T., D. Kruse, S. Zhu, K. R. Seshan, and R. W. Wheat.** 1990. Composition, serologic reactivity, and immunolocalization of a 120-kilodalton tube precipitin antigen of *Coccidioides immitis*. Infect. Immun. **58**:179–188.
7. **Cole, G. T., L. M. Pope, M. Huppert, S. H. Sun, and P. Starr.** 1983. Ultrastructure and composition of conidial wall fractions of *Coccidioides immitis*. Exp. Mycol. **7**:297–318.
8. **Cole, G. T., S. Zhu, S. Pan, L. Yuan, D. Kruse, and S. H. Sun.** 1989. Isolation of antigens with proteolytic activity from *Coccidioides immitis*. Infect. Immun. **57**:1524–1534.
9. **Cox, R. A., and L. A. Britt.** 1986. Isolation of a coccidioidin component that reacts with immunoglobulin M precipitin antibody. Infect. Immun. **53**:449–453.
10. **Cox, R. A., and L. A. Britt.** 1987. Antigenic identity of biologically active antigens in coccidioidin and spherulin. Infect. Immun. **55**:2590–2596.
11. **Cox, R. A., M. Huppert, P. Starr, and L. A. Britt.** 1984. Reactivity of an alkali-soluble, water-soluble antigen of *Coccidioides immitis* with anti-*Coccidioides* immunoglobulin M precipitin antibody. Infect. Immun. **43**:502–507.
12. **Decker, L. A. (ed.).** 1977. Worthington enzyme manual. Worthington Biochemical Corp., Freehold, N.J.
13. **Dolan, M. J., R. A. Cox, V. Williams, and S. Woolley.** 1989. Development and characterization of a monoclonal antibody against the tube precipitin antigen of *Coccidioides immitis*. Infect. Immun. **57**:1035–1039.
14. **Einstein, H., and A. Catanzaro (ed.).** 1985. Coccidioidomycosis. Proceedings of the Fourth International Symposium on Coccidioidomycosis. National Foundation for Infectious Diseases, Washington, D.C.
15. **Galgiani, J. N., K. O. Dugger, N. M. Ampel, S. H. Sun, and J. H. Law.** 1988. Extraction of serologic and delayed hypersensitivity antigens from spherules of *Coccidioides immitis*. Diagn. Microbiol. Infect. Dis. **11**:65–80.
16. **Goldstein, I. J., C. M. Reichert, and A. Misaki.** 1974. Interactions of concanavalin A with model substrates. Ann. N.Y. Acad. Sci. **234**:283–296.
17. **Huppert, M., and J. W. Bailey.** 1963. Immunodiffusion as a screening test for coccidioidomycosis serology. Sabouraudia **2**:284–291.
18. **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.
19. **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.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Millette, C. F., and B. K. Scott.** 1984. Identification of spermatogenic cell plasma membrane glycoproteins by two-dimensional electrophoresis and lectin blotting. J. Cell Sci. **65**:233–248.
22. **Pappagianis, D., C. E. Smith, G. S. Kobayashi, and M. J. Saito.** 1961. Studies of antigens from young mycelia of *Coccidioides immitis*. J. Infect. Dis. **108**:35–44.
23. **Rabilloud, T., G. Carpentier, and P. Tarroux.** 1988. Improvement and simplification of low-background silver staining of proteins by sodium dithionate. Electrophoresis **9**:288–291.
24. **Reeke, G. N., J. W. Becker, B. A. Cunningham, G. R. Gunther, J. L. Wang, and G. M. Edelman.** 1974. Relationships between the structure and activities of concanavalin A. Ann. N.Y. Acad. Sci. **234**:369–382.
25. **Rippon, J. W.** 1988. Medical mycology: the pathogenic fungi and the pathogenic actinomycetes, 3rd ed, p. 433–473. The W. B. Saunders Co., Philadelphia.
26. **Smith, C. E., M. T. Saito, R. R. Beard, R. M. Kepp, R. W. Clark, and B. U. Eddie.** 1950. Serological tests in the diagnosis and prognosis of coccidioidomycosis. Am. J. Hyg. **52**:1–21.
27. **Smith, C. E., M. T. Saito, and S. A. Simons.** 1956. Pattern of 39,500 serologic tests in coccidioidomycosis. J. Am. Med. Assoc. **160**:546–552.
28. **Smith, C. E., E. G. Whiting, E. E. Baker, H. G. Rosenberger, R. R. Beard, and M. T. Saito.** 1948. The use of coccidioidin. Am. Rev. Tuberculosis **57**:330–360.
29. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
30. **Yuan, L., and G. T. Cole.** 1987. Isolation and characterization of an extracellular proteinase of *Coccidioides immitis*. Infect. Immun. **55**:1970–1978.
31. **Zimmer, B. L., and D. Pappagianis.** 1988. Characterization of a soluble protein of *Coccidioides immitis* with activity as an immunodiffusion-complement fixation antigen. J. Clin. Microbiol. **26**:2250–2256.
32. **Zimmer, B. L., and D. Pappagianis.** 1989. Immunoaffinity isolation and partial characterization of the *Coccidioides immitis* antigen detected by the tube precipitin and immunodiffusion-tube precipitin tests. J. Clin. Microbiol. **27**:1759–1766.