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# Development and Characterization of a Monoclonal Antibody against the Tube Precipitin Antigen of *Coccidioides immitis*

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**Primary infection with *Coccidioides immitis* is commonly accompanied by the production of an immunoglobulin M precipitin antibody which is detected by the tube precipitin (TP) assay or by the immunodiffusion assay for TP antibody (IDTP assay). In the present investigation, spleen cells from spherulin-immunized BALB/c mice were fused with SP2/O Ag14 myeloma cells, and the resulting hybridomas were screened for antibody to the IDTP antigen by using an enzyme-linked immunosorbent assay. Positive hybridomas were cloned by limiting dilution and injected into pristane-primed mice for ascites production. Characterization of antibody reactivity was accomplished with the IDTP assay, two-dimensional immunoelectrophoresis, and immunoblotting. An immunoglobulin G1 monoclonal antibody which reacts with the IDTP antigen of *C. immitis* is described. The epitope that is recognized by the monoclonal antibody is also present, but to a lesser extent, on a second coccidioidal antigen which has been designated antigen 2. The monoclonal antibody was not reactive in immunoblots of histoplasmin or blastomycin, indicating that the epitope recognized by this antibody may be specific for *C. immitis*.**

*Coccidioides immitis* is a dimorphic fungus that has a well-described pathogenicity, ranging from an acute self-limited pneumonitis to a progressive, disseminated, and often fatal disease (5). Primary infection is commonly associated with the development of an immunoglobulin M (IgM) precipitin antibody which can be detected by the classical tube precipitin (TP) test developed by Smith and colleagues (15) or an immunodiffusion assay for TP antibody (IDTP assay) developed by Huppert and co-workers (9, 10). Although the TP and IDTP assays are useful serologic tests, they lack sensitivity compared with other immunoassay procedures, such as an enzyme-linked immunosorbent assay (ELISA) or immunoblot assay. Development of a more sensitive immunoassay for detecting this antibody response is compromised, however, by the lack of monospecific reference reagents.

We previously reported the purification of the IDTP antigen from coccidioidin (CDN) by using a combination of immunoaffinity and ion-exchange chromatography (2). Immunizations of goats with the purified IDTP antigen were unsuccessful, or the antibody response was weak and short-lived. The present study was undertaken, therefore, to produce a stable hybridoma to the IDTP antigen.

## MATERIALS AND METHODS

**Antigens and antisera.** CDN and spherulin (SPH) were prepared as toluene-induced lysates of mycelial- and spherule-phase cells, respectively, of *C. immitis* Silveira grown in modified Converse medium (3). The IDTP antigen was purified from CDN by using a combination of immunoaffinity chromatography and ion-exchange chromatography (2). The purified antigen was reactive in the IDTP assay (Meridian Diagnostics, Inc., Cincinnati, Ohio) and was antigenically homogeneous when evaluated by two-dimensional immunoelectrophoresis (2D-IEP) against hyperimmune goat antisera to CDN and SPH.

The goat antisera to *C. immitis* antigens were the same preparations as those used in previous studies (2, 3). The immunoglobulins were isolated by precipitation of the sera with an equal volume of saturated ammonium sulfate, and the precipitated immunoglobulin fractions were dialyzed against distilled water and then lyophilized. Histoplasmin, blastomycin, and polyvalent rabbit antisera to these antigens were purchased from Nolan Laboratories (Atlanta, Ga.).

Affinity-purified alkaline phosphatase-conjugated rabbit anti-goat (directed against the heavy and light chains of IgG), goat anti-rabbit (directed against IgM, IgG, and IgA), and goat anti-mouse (Fc specific) IgG were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, Md.).

**Immunization of mice.** SPH was admixed with incomplete Freund adjuvant and used to immunize 5- to 10-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, Maine). Mice were injected with 100 μg of SPH in adjuvant by the intramuscular or subcutaneous route. The injections were given over a period of 90 days, and a final injection of 400 μg of SPH (without adjuvant) was given intravenously at 3 days before hybridization. Sera were screened for antibody against the purified IDTP antigen by the ELISA described below. Mice exhibiting antibody reactivity at a 1:100 dilution of serum were used as a source of spleen cells for fusion with myeloma cells.

**Production of monoclonal antibodies (MAb).** Fusion of $1.4 \times 10^8$ immune spleen cells with $7 \times 10^7$ log-phase Sp2/O Ag14 murine myeloma cells (American Type Culture Collection, Rockville, Md.) was accomplished with 1.5 ml of 40% polyethylene glycol 1450 as described by Gefter et al. (6). Fusion products were plated on 96-well flat-bottom microdilution plates containing $1 \times 10^5$ normal BALB/c spleen cells in 200 μl of hypoxanthine-aminopterin-thymidine hybrid-selective medium. Resulting hybridomas were initially screened with the ELISA in which SPH was the target antigen. Positive hybridomas were then evaluated by the ELISA for reactivity against the purified IDTP antigen. Hybridomas with antibody specificity to the IDTP antigen were cloned twice by limiting dilution and then injected intraperitoneally into pristane-primed BALB/c mice for the

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production of ascites. The isotypes were determined by using a commercial ELISA procedure (Zymed, South San Francisco, Calif.).

**ELISA.** The ELISA was performed according to the method of Voller and Bidwell (17). Antigen was suspended in 0.1 M bicarbonate buffer (pH 9.6) to a concentration of 10 μg/ml. Samples of 0.1 ml were added to polystyrene microdilution wells (Immunolon II; Dynatech Laboratories, Inc., Alexandria, Va.), and the plates were incubated overnight at 4°C. The wells were blocked by the addition of 1% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20 (PBS-TW; pH 7.4). The plates were washed twice in PBS-TW, and the wells were reacted with 100 μl of serum, diluted 1:100 in PBS-TW, or with 100 μl of hybridoma supernatant, diluted 1:2 in PBS-TW. After a 2-h incubation at 37°C, the plates were washed and 100 μl of alkaline phosphatase-conjugated goat anti-mouse IgG, diluted 1:1,000 in PBS-TW, was added. The plates were incubated for 90 min at 37°C and washed with PBS-TW, and substrate (*p*-nitrophenyl phosphate diluted in 10% diethanolamine buffer, pH 9.8) was added. Enzymatic reactions were terminated 1 h later by the addition of 50 μl of 3 M NaOH, and the $A_{410}$ were read.

**2D-IEP.** The techniques of 2D-IEP were performed as described previously (2–4, 11). For intermediate-gel 2D-IEP, CDN was electrophoresed in the first dimension for 1 h at 10 V/cm. An agarose strip (width, 7 mm) containing MAb or, for a control, ascites fluid from pristane-treated BALB/c mice injected with the SP2/O Ag14 myeloma cell line was poured parallel to the first-dimension gel. The intermediate gel was allowed to solidify, and agarose containing goat anti-CDN was applied to the remainder of the second-dimension gel area. The gel was then electrophoresed overnight at 3 V/cm, in a direction perpendicular to the first axis.

Radioimmunoelectrophoresis was performed as described by Globe et al. (7). The MAb was affinity purified on an Affi-Gel Protein A MAPS II column (Bio-Rad Laboratories, Richmond, Calif.) and radiolabeled with $^{125}I$ (specific activity, 14.8 mCi/μg; Amersham Corp., Arlington Heights, Ill.), using *N*-chlorobenzenesulfonamide-derivatized polystyrene beads (IODO-BEADS; Pierce Chemical Co., Rockford, Ill.). The $^{125}I$-labeled MAb was recovered from the mixture by passage over a Sephacryl 300 column (1 by 27 cm) preequilibrated with 0.0067 M potassium phosphate buffer (pH 7.4). For 2D-IEP, the radiolabeled MAb (specific activity, 0.2 μCi/μg) was admixed with a reference hyperimmune goat antiserum and the antibody mixture was incorporated into the second-dimension gel. After electrophoresis, the gels were washed, dried, and placed in direct contact with Cronex high-speed X-ray film (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) for 72 h at −70°C. The gels were removed and stained with Coomassie blue so that the precipitin peaks developed by the polyvalent antiserum could be aligned with those visualized by autoradiography.

**Immunoblot analysis.** Immunoblotting was performed as previously reported (4). Antigens were diluted in buffer containing 1% sodium dodecyl sulfate, 2.5% 2-mercaptoethanol, 0.001% bromophenol blue, 0.0625 M Tris hydrochloride, and 5% glycerol and then heated for 5 min in a boiling water bath. The samples were electrophoresed through a discontinuous 4 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel under reducing conditions. The separated antigens were then electrophoretically transferred to nitrocellulose acetate membranes at 0.6 A overnight (16). The membranes were blocked with Bovine Lacto Transfer Technique Optimizer (Hoefer Scientific Instruments, San Francisco, Calif.) and then reacted with goat or rabbit polyclonal antisera or MAb overnight at room temperature. As a negative control for MAb to *C. immitis* antigen, replicate lanes were probed with an irrelevant MAb directed against alpha-tubulin (Cederlane Laboratories, Westburg, N.Y.). After incubation, the membranes were washed in Bovine Lacto Transfer Technique Optimizer and individual lanes were reacted for 1 h at 25°C with alkaline phosphatase-conjugated rabbit anti-goat IgG, goat anti-rabbit IgG, or goat anti-mouse IgG, depending on the primary antibody used. The blots were washed three times in phosphate-buffered saline and developed with 5-bromo-4-chloro-3-indolyl-phosphate–Nitro Blue Tetrazolium (BCIP-NBT phosphatase substrate; Kirkegaard and Perry), diluted 1:10 in 0.1 M Tris buffer (pH 9.5).

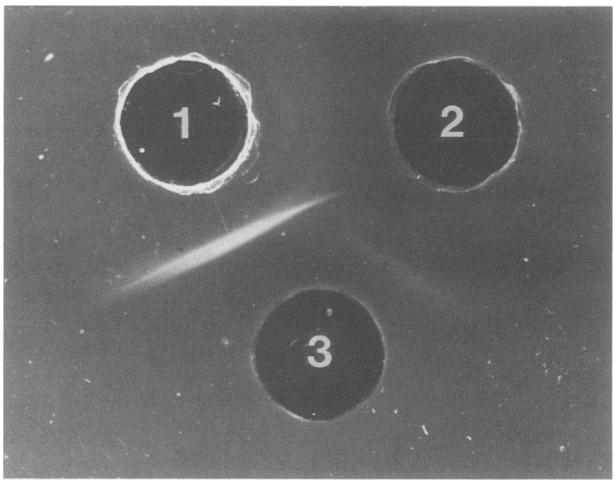


FIG. 1. Reactivity of MAb in the IDTP assay. Well 1, Reference anti-IDTP serum; well 2, MAb (as undiluted ascites); well 3, reference IDTP antigen.

The molecular size distribution of bands reactive with MAb or polyvalent antisera was determined by coelectrophoresis of molecular size standards (Pharmacia, Uppsala, Sweden). These consisted of thyroglobulin (330 kilodaltons [kDa]), ferritin (220 kDa), β-galactosidase (116 kDa), phosphorylase *b* (94 kDa), albumin (67 kDa), and carbonic anhydrase (30 kDa).

## RESULTS

**Reactivity of MAb in IDTP assay.** Of 10 mice immunized with SPH, sera from 2 were reactive to the IDTP antigen when assayed by the ELISA. Hybridomas prepared from the fusions of spleen cells from these two mice yielded five MAb of the IgG isotype. Only one of these, an IgG1 MAb, demonstrated precipitin activity in the IDTP assay (Fig. 1). The precipitin line formed in the reaction between the MAb and reference IDTP antigen was of partial identity with the band obtained between the reference IDTP antigen-antiserum system. This pattern of partial identity would be consistent with the diverse epitope-antibody interactions with the titrated polyclonal goat anti-IDTP serum as opposed to the monospecificity of the MAb (13).

**Reactivity of MAb in 2D-IEP.** We have previously reported that the IDTP antigen is characterized as an incomplete precipitinogen in 2D-IEP by having a cathodal precipitin leg and a partial anodal leg (2). The cathodal leg of the IDTP antigen parallels the cathodal leg of the major precipitin peak

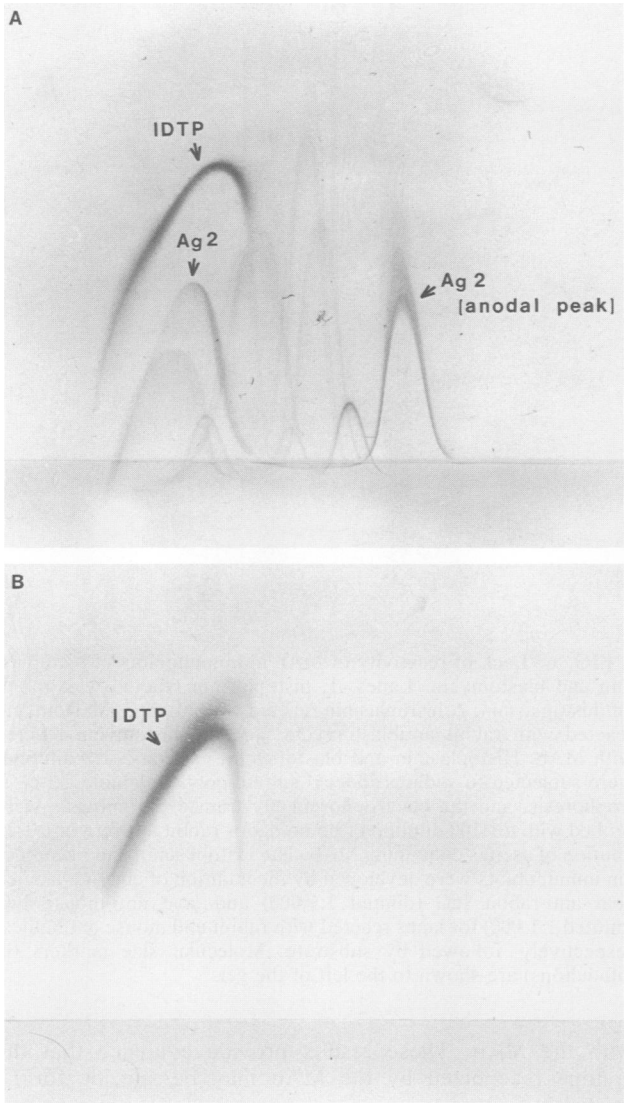


FIG. 2. Reactivity of MAb in 2D-IEP of CDN. CDN (400 μg) was electrophoresed in the first-dimension gel and then against a second-dimension gel containing goat anti-CDN (400 μg/cm$^2$ of gel) (A) or MAb (ascites diluted 1:5) (B).

of a polymeric component previously designated antigen 2 (Ag2) by Huppert et al. (11) (Fig. 2A).

When the MAb was assayed by 2D-IEP against CDN, only the IDTP antigen was precipitated (Fig. 2B). Identical results were obtained by 2D-IEP of SPH (results not shown). However, incorporation of the MAb in a gel interposed between the first-dimension electrophoresis of CDN (or SPH) and homologous goat antiserum effected a slight reduction of the height of the cathodal peak of the Ag2 polymer, as well as an increased staining pattern and reduction in the precipitin peak of the IDTP antigen (compare Fig. 3A and B). This combined effect would be consistent with the MAb recognizing an epitope which is common to the IDTP antigen and Ag2 but present in lower concentrations on the latter antigen. An alternative explanation would be that Ag2 and the IDTP antigen are antigenically distinct but physically associated, so that a reduction in the peak height of the IDTP antigen would be accompanied by a reduction in the peak height of Ag2.

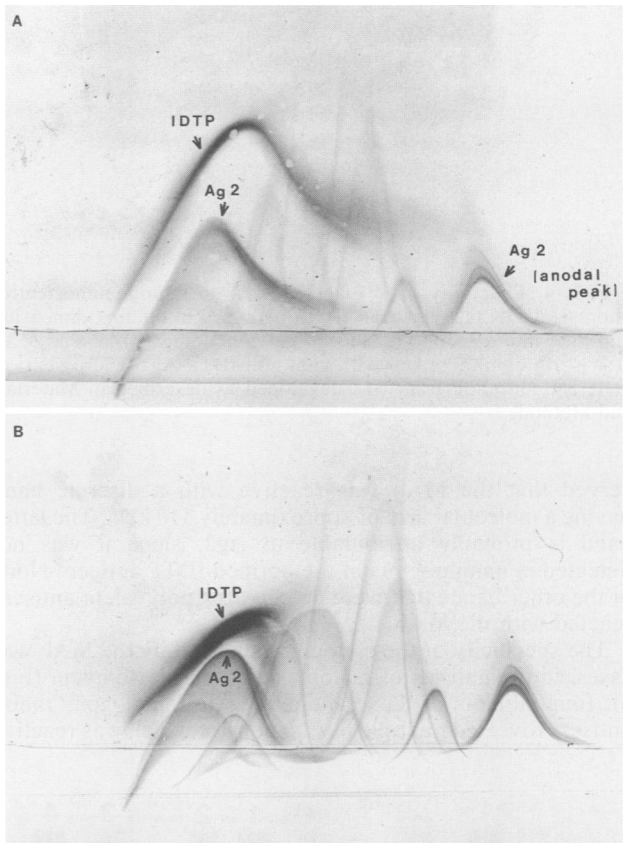


FIG. 3. Reactivity of MAb in intermediate-gel 2D-IEP. An intermediate gel containing control ascites, produced by injecting BALB/c mice intraperitoneally with Sp2/O Ag14 myeloma cells (A), or ascites containing MAb (B) was interposed between the first-dimension electrophoresis gel of CDN (400 μg) and a second-dimension gel containing goat anti-CDN (453 μg/cm$^2$ of gel). Both the control and MAb-containing ascites were assayed at a 1:2 dilution in agarose.

To distinguish among these possibilities, we used a combined technique of 2D-IEP and autoradiography in which $^{125}$I-labeled MAb was admixed with goat antiserum to Ag2 and the IDTP antigen. The inclusion of the polyvalent antiserum served to develop the precipitin pattern of Ag2, since the MAb alone did not precipitate this antigen. The radiolabeled MAb demonstrated reactivity with both the IDTP antigen and Ag2 (Fig. 4). These results provide definitive evidence that the MAb recognizes an epitope which is common to the IDTP antigen and Ag2.

**Reactivity of MAb in immunoblots.** Immunoblotting was used to assess the molecular size distribution of the IDTP antigen and to further characterize the reactivity of the MAb. The results obtained in immunoblots of the purified IDTP antigen against the MAb and, for comparison, against goat anti-CDN are shown in Fig. 5A. Both the polyclonal antiserum and the MAb detected a diffusely staining band with a molecular size distribution of 130 to 330 kDa. No reactivity was observed when the immunoblots of the IDTP antigen were reacted with an irrelevant IgG1 MAb against alpha-tubulin (results not shown).

When immunoblots of CDN and SPH were probed with the MAb, a diffusely staining band with a molecular size range corresponding to that obtained with the purified IDTP antigen was detected (Fig. 5B). We also consistently ob-

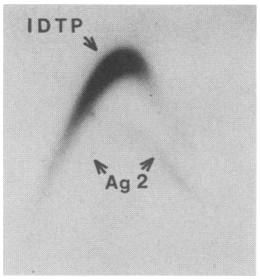


FIG. 4. Reactivity of $^{125}$I-labeled MAb in radioimmunoelectrophoresis. CDN (400 μg) was electrophoresed in the first dimension and then into a second-dimension gel containing $^{125}$I-labeled MAb ($1.4 \times 10^6$ cpm) admixed with goat anti-IDTP-Ag2 (800 μg/cm$^2$ of gel). The autoradiogram was developed as described in Materials and Methods.

served that the MAb was reactive with a discrete band having a molecular size of approximately 110 kDa. The latter band is probably attributable to Ag2, since it was not detected in immunoblots of the purified IDTP antigen. None of the other bands that were detected by polyvalent antisera reacted with the MAb.

The specificity of the epitope recognized by the MAb was assessed in analyses of histoplasmin and blastomycin (Fig. 6). Immunoblots of these antigens with homologous rabbit antisera revealed multiple bands, none of which was reactive with the MAb. These results provide evidence that the epitope recognized by the MAb may be specific for *C. immitis*.

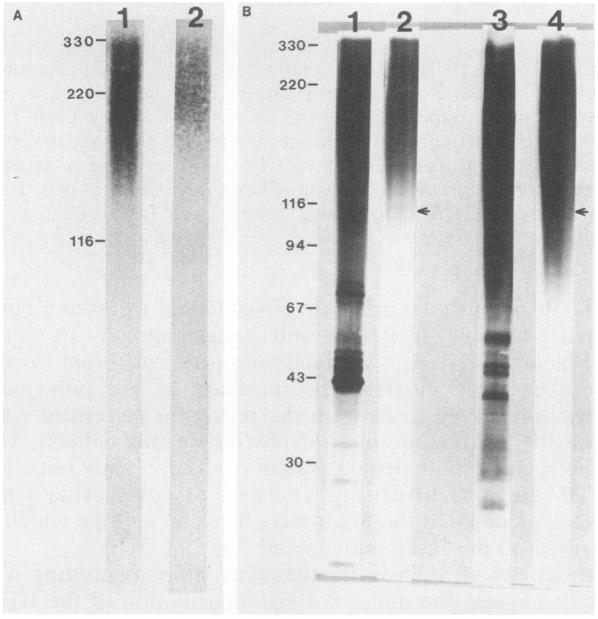


FIG. 5. Reactivity of the MAb in immunoblots of *C. immitis* antigens. Antigens were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose acetate membranes, and reacted with MAb or polyclonal goat antisera followed by enzyme-labeled goat anti-mouse (1:1,000) or rabbit anti-goat (1:1,000) IgG, respectively, and then substrate. (A) Purified IDTP antigen (38 μg) was probed with goat anti-CDN (150 μg/ml) (lane 1) and with MAb (1:25 dilution of ascites) (lane 2). (B) CDN (38 μg) was probed with goat anti-CDN (30 μg/ml) (lane 1) and MAb (ascites diluted 1:25) (lane 2). SPH (38 μg) was probed with goat anti-SPH (10 μg/ml) (lane 3) and MAb (ascites diluted 1:25) (lane 4). The arrow identifies the discrete band in CDN and SPH that also reacts with the MAb. Molecular size markers (in kilodaltons) are shown to the left of the gels.

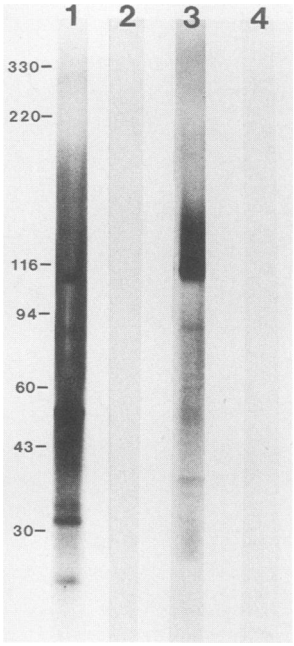


FIG. 6. Lack of reactivity of MAb in immunoblots of histoplasmin and blastomycin. Lanes: 1, histoplasmin reacted with rabbit antihistoplasmin; 2, histoplasmin reacted with MAb; 3, blastomycin reacted with rabbit antiblastomycin; and 4, blastomycin reacted with MAb. Histoplasmin and blastomycin, each at a 1:2 dilution, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the electrophoretically transferred antigens were probed with a 1:100 dilution of homologous rabbit antisera or a 1:25 dilution of ascites containing MAb. The histoplasmin and blastomycin immunoblots were developed by the addition of enzyme-labeled goat anti-rabbit IgG (diluted 1:1,000) and goat anti-mouse IgG (diluted 1:1,000) for lanes reacted with rabbit and mouse antibodies, respectively, followed by substrate. Molecular size markers (in kilodaltons) are shown to the left of the gel.

## DISCUSSION

Human antibody response to the TP antigen characteristically follows a course of early rise in IgM titer, which diminishes to nondetectable levels within 4 months of clinical onset (15). In a recent study (2), we reported the isolation of the antigen that reacts with this antibody, as measured by the TP and IDTP assays. The antigen was shown to be resistant to heat treatment and proteolytic digestion but susceptible to periodate oxidation, indicating a predominant polysaccharide composition (2). In the present investigation, we report the production of an IgG1 MAb directed against this antigen. Although the MAb exhibits precipitin reactivity against the IDTP antigen, the precipitin band formed is one of partial identity with that of the reference IDTP antiserum-antigen system. This partial identity is consistent with the recognition of multiple epitopes by the polyclonal antiserum as opposed to the monospecificity of the MAb (13).

The molecular size distribution of the IDTP antigen, as estimated by immunoblotting against the MAb or hyperimmune goat antiserum, ranges from 130 to 330 kDa. In two previous reports, investigators have utilized IDTP-positive sera from coccidioidomycosis patients to identify this anti-

gen in immunoblots of *C. immitis* extracts (1, 18). Calhoun et al. (1) demonstrated reactivity of IgM antibody(s) with a broad band having a molecular size distribution of 80 to 120 kDa. At variance with this finding and with the results obtained in this study, Zimmer and Pappagianis (18) reported IgM reactivity with doublet bands in the 50- to 65-kDa range. These divergent molecular size ranges may be attributable to the differences in antigen preparations or in the methods used for immunoblotting. It bears emphasizing, however, that electrophoretic techniques are not suitable for assigning molecular sizes to antigens having predominant polysaccharide compositions (14).

The epitope recognized by the MAb is not unique to the IDTP antigen but is also present on a polymeric antigen which has been designated Ag2 (11). The distribution (or exposure) of the epitope is quantitatively greater on the IDTP antigen than on Ag2. This conclusion is based on the finding that the MAb did not exhibit precipitin reactivity against Ag2 and that, when evaluated by radioimmunoelectrophoresis, the $^{125}$I-labeled MAb was only weakly reactive with Ag2. Of further note, the reactivity of the MAb with Ag2 appears to be limited to the cathodal precipitin peak of this polymeric antigen. This is concordant with our earlier report that IDTP determinants were distributed throughout the cathodal peak but not the anodal precipitin peak of Ag2, as assessed by line IEP of the purified IDTP antigen in a gel interposed between CDN and goat anti-CDN (2).

The specificity of the MAb was evaluated in immunoblots of histoplasmin and blastomycin. No reactivity was demonstrable, indicating that the epitope recognized by this MAb may be specific for *C. immitis*. Recent studies by Kaufman and co-workers (12) have established, however, that the IDTP antigen is of antigenic identity with a precipitinogen produced by certain arthroconidia-forming saprophytes, such as *Malbranchea, Arachniotus,* and *Auxarthron* species. Hence, conclusions regarding the specificity of the IDTP epitope recognized by the MAb await analyses of extracts from these gymnoascaceous fungi.

Our finding that the MAb is reactive with both the IDTP antigen and Ag2 precludes its utility as a ligand for purifying the IDTP antigen. Studies are in progress, however, to determine whether the MAb would be useful as a probe for detecting circulating antigen in patients with coccidioidal disease and to assess the effect of passively transferred MAb on the pathogenesis of experimental murine coccidioidomycosis.

## ACKNOWLEDGMENT

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