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Antigenemia Detected in Human Coccidioidomycosis

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To advance the serological diagnosis of coccidioidomycosis, a radioimmunoassay was developed to detect an antigen of *Coccidioides immitis* and evaluated with serum samples from patients and controls. The assay antigen was fractionated from a culture filtrate of *C. immitis* cells by Sephacryl S300 gel chromatography. The assay used preliminary acid-heat extraction to dissociate serum-bound antigen and staphylococcal protein A as an absorbent. Antigenemia was detected in five (56%) of nine patients with coccidioidomycosis. Three of four negative tests occurred with sera from patients who had already received antifungal chemotherapy. Antigenemia was not detected in 106 control patients and normal donors. One false-positive reaction was observed in a patient with histoplasmosis. In all nine patients with active coccidioidomycosis, anti-*C. immitis* antibodies were detected during the initial evaluation or in the follow-up. In summary, an immunoassay for *C. immitis* antigenemia was developed; antigenemia was detected in five (56%) of nine patients with active coccidioidomycosis and a positive test indicated active or persistent disease.

Coccidioidomycosis, which occurs endemically in areas of the Southwest, is believed to affect 100,000 Americans each year (3). To date, only tests to detect patient antibody have been used in the serodiagnosis of coccidioidomycosis. An alternative approach to serological diagnosis is the detection of fungal antigen in serum and other body fluids. There are two advantages to this approach: a direct correlation may be established between antigenemia and disease activity, and the assay result does not depend on the humoral response of the patient, which can be attenuated or delayed. For two other systemic mycoses, candidiasis (19, 21) and aspergillosis (18, 20), the presence of antigenemia can be used for early, specific serological diagnosis.

The aim of the present study was to determine whether fungal antigenemia also occurs in coccidioidomycosis and, if so, to evaluate its diagnostic significance. For the study, a complex antigenic mixture of *Coccidioides immitis* cells was fractionated, and an antigen which is an immunogen in clinical infection was isolated. A radioimmunoassay (RIA) to this *C. immitis* antigen was then developed and evaluated by using sera from patients with mycotic and bacterial infections and from normal donors.

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MATERIALS AND METHODS

Patients. The sera to be tested were drawn from patients with suspected fungal infections, patients hospitalized for other reasons, and normal donors. The diagnosis of coccidioidomycosis was established by isolating *C. immitis* from sputum, cerebrospinal fluid, bone marrow, or fistulous exudate samples. Sera were also available from nine patients with a diagnosis of active disease; seven of these patients had received antifungal chemotherapy before the serological study. The sera were frozen at −20°C with 0.1% sodium azide. Informed consent was obtained from both patients and normal donors.

Immunochemical techniques. Double immunodiffusion precipitation and crossed immunoelectrophoresis were performed by the methods of Ouchterlony (15) and Axelsen (2), respectively. All of the gel precipitin systems used 2% agar (Marine Colloids; Rockland, Maine) in veronal buffer, 0.05 ionic strength (pH 8.4). Immunoabsorbent columns were prepared by the method of Porath et al. (16). Analytical polyacrylamide gel electrophoresis was carried out in 13% Neville gels with a discontinuous buffer system (14). Sodium dodecyl sulfate was not included in the gel. Electrophoresis was carried out at 50 V until the sample entered the stacking well-forming gel and at 150 V thereafter. The gels were stained overnight in 0.03% Coomassie brilliant blue R250–10% acetic acid–

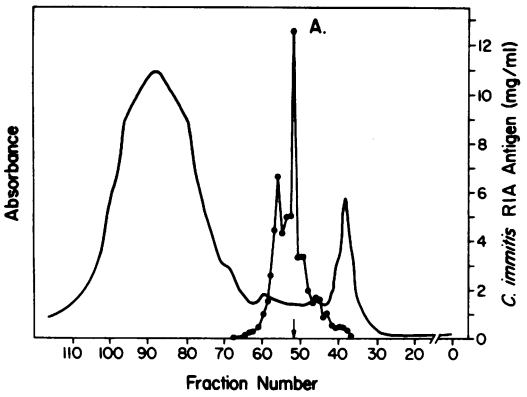

FIG. 1. Chromatography of the *C. immitis* culture filtrate over Sephacryl S300 superfine gel. The void volume (solid line) was indicated by blue dextran absorbance at 254 nm. ●, *C. immitis* antigen detected by the RIA in the chromatogram. A, Major antigenic peak detected by the RIA in fraction 52. Chromatogram fractions were run by double immunodiffusion (see Fig. 3), crossed immunoelectrophoresis (see Fig. 4), and polyacrylamide gel electrophoresis (see Fig. 5).

50% methanol and then destained by diffusion in 7% acetic acid–10% methanol. Periodic acid-Schiff staining of the gels was performed by the method of Hebert and Strobbel (6). Isoelectric focusing (22) was done in 5% polyacrylamide gels (pH 3.5 to 9.5) (Ampholine PAGplate; LKB Instruments, Inc., Rockville, Md.). Carbohydrate determinations were done by the phenol-sulfuric acid colorimetric method (7), and protein was estimated by the method of Lowry et al. (13). Chromatograhic fractions of the coccidioidin culture filtrate were mildly oxidated with 0.01 M periodate at 22°C for 1 h.

Antisera. New Zealand white rabbits were injected monthly with the coccidioidin culture filtrate emulsified in Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). The animals were bled at week 9 and every month thereafter. Serum samples were stored at −80°C with 0.1% sodium azide.

Preparation of RIA antigen. Coccidioidin from the culture filtrate of six strains of *C. immitis* (11) and reference anticoccidioidin antisera (10) were generously supplied by Milton Huppert, Audie L. Murphy Memorial Veterans Hospital. The *C. immitis* culture filtrates were concentrated sevenfold by ultrafiltration with a PM 10 Diaflo membrane (Amicon Corp., Lexington, Mass.). After concentration, the coccidioidin was fractionated by gel filtration over Sephacryl S300 superfine gel (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) under the following conditions: bed dimensions, 2.6 by 100 cm; flow rate, 36 ml/h; eluant, 0.10 M phosphate buffer (pH 7.4)–0.9% sodium chloride. The protein content was detected by absorbance at 254 nm. Antigenic analysis was performed initially by double immunodiffusion and then by RIA (Fig. 1). An immunoreactive pool of fractions with molecular weights between 200,000 and 230,000 was dialyzed, concentrated by ultrafiltration, and conjugated to tyramine with cyanogen bromide (16). The conjugated *C. immitis* antigen (35 μg of protein) was radiolabeled with ^{125}I, with 50 μg of chloramine-T used as the oxidizing agent (9). The radiolabeled antigen was fractionated by gel filtration over Sephadex G-10 (Pharmacia Fine Chemicals) and then eluted from a coccidioidin antibody solid-phase immunoabsorbent with 0.1 M glycine (pH 3). The efficiency of ^{125}I incorporation in six consecutive experiments was 51.5 ± 6.1% (± standard error of the mean), and the specific activity of the radiolabeled antigen was 31.8 ± 3.7 μCi/μg of protein. The labeled antigen was 82% precipitable with excess antisera and was stable for more than 2 weeks.

RIA procedures. Duplicate assays for each sample were performed as follows. Sera diluted 1:1 in normal saline (300 μl of each) were acidified with 600 μl of 0.04 M citric acid (pH 2.7), heated at 96°C for 20 min, and clarified by centrifugation at 3,900 × *g* for 12 min. Four 100-μl replicate samples of the supernatant were neutralized in 100 μl of 0.35 M phosphate buffer (pH 8.96). The neutralized sera were incubated at 20°C for 1 h with 100 μl of *C. immitis* antiserum diluted in RIB (0.1 Trizma buffer [Sigma Chemical Co., St. Louis, Mo.], pH 7.3–0.9% NaCl–2% bovine serum albumen–0.1% sodium azide) to bind 25 to 35% of counts in the absence of inhibiting *C. immitis* antigen. The sera then were incubated at 4°C for 14 h with ^{125}I-labeled antigen (6,000 cpm) diluted in RIB. The next day, 20 μl of beads containing staphylococcal protein A was added to each tube, and the tubes were incubated for 45 min. The supernatants were aspirated, and the pellets were counted in a gamma counter. Standards for the RIA were made by diluting the coccidioidin culture filtrate in normal sera. Standard curves were derived from linear transformation of raw counts in the standards as described by Feldman and Rodbard (4) and adapted by Groner et al. (5). Assays were rejected if the correlation coefficient of the standard curve was <0.95. The concentration of coccidioidin antigen in the unknowns was calculated from the standard curve when the ratio of counts bound to counts bound at zero concentration (B/B_o) was <0.94 and the logit (B/B_o) was <2.75. The

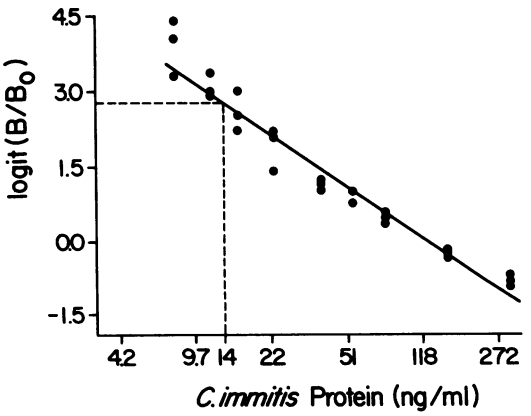

FIG. 2. Linear transformation of a standard curve, in which the logit (B/B_o) is plotted against nanograms of protein per milliliters of coccidioidin culture filtrate. *B* is the counts of radiolabeled antigen bound in the standards, and B_o is the radiolabeled counts bound in the absence of unlabeled antigen.

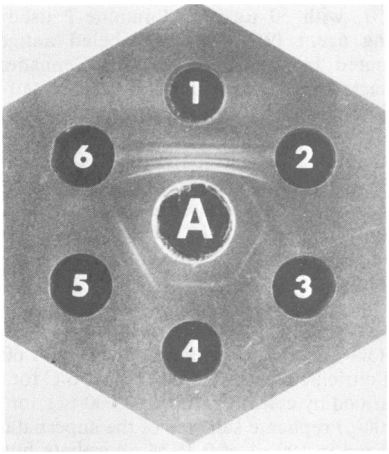

FIG. 3. Double immunodiffusion plate, with the coccidioidin culture filtrate in well 1 and the reference polyvalent antiserum in the center well. The *C. immitis* antigen preparation in well 2, used for radiolabeling in the RIA, formed a line of identity with pooled chromatography fractions 45 to 49 (well 3) and 50 to 56 (well 4) obtained by gel filtration over Sephacryl S300 (see Fig. 1). Pooled chromatography fractions 63 to 71 were placed in well 5. The immunological activity of the RIA antigen was removed by mild periodate oxidation (well 6).

antigen concentration in the unknowns was expressed as nanograms of protein per milliliter of coccidioidin culture filtrate. A representative standard curve expressing the logit (B/B_0) to (concentration standard) transformation is shown in Fig. 2.

The sensitivity of the RIA was 14 ng of protein per ml of *C. immitis* culture filtrate. The assay was specific when tested with other isolated microbial carbohydrates. Mannans from *Candida albicans, Candida stellatoidea, Saccharomyces cerevisiae* and *Aspergillus fumigatus* cell wall carbohydrates, and dextran, dextrose, and mannose at a concentration of 200 μg/ml were not cross-reactive in the RIA. The precision of the RIA was determined by testing a single serum 25 times. The coefficient of variation of the uncorrected counts was 2.20. The accuracy of the RIA was determined by preparing 25 sera with coccidioidin protein at a concentration of 40 ng/ml. The mean concentration determined by the RIA was 35.4 ± 1.3 ng/ml (± standard error of the mean).

RIAs for candidal and *A. fumigatus* carbohydrates were performed as previously described (18, 19).

Coccidioides antibody. *C. immitis* complement-fixing antibody determinations were performed by the Texas Department of Health Laboratories, and the immunodiffusion complement-fixing and precipitin antibodies (11, 12) were determined by J. Johnson, Audie L. Murphy Memorial Veterans Hospital.

RESULTS

The coccidioidin culture filtrate was initially fractionated by gel filtration. In pilot studies, antigenemia was detected by RIA to the compound(s) contained in a pool of chromatography fractions whose molecular weights ranged from 140,000 to 240,000. This preparation contained three components when characterized by double immunodiffusion and nine components when examined by thin-layer polyacrylamide gel isoelectric focusing (pK_a range, 2.5 to 4.8).

From this initial preparation, an RIA was developed to a component identified by immunoprecipitin assays (Fig. 3). For the RIA, an antigen was partially purified from the coccidioidin culture filtrate by chromatography over Sephacryl S300 gel (Fig. 1). A preparation of fractions with molecular weights between 200,000 and 230,000, which included peak A but not the other minor peaks, was used. When characterized by crossed immunoelectrophoresis, a preparation of the RIA-reactive antigen (Fig. 1, peak A) migrated to the anode in a broad arc (Figure 4). When examined by polyacrylamide gel electrophoresis (Figure 5) and isoelectric focusing, the RIA antigen was not sharply resolved. In an analysis by RIA of the Sephacryl S300 chromatographic fractions, antigenic activity was detected in at least two peaks. The activity detected by RIA and by double immunodiffusion was concordant over the range of molecular weights tested from the gel chromatograms. Finally, the RIA antigen appeared to be a protein-carbohydrate conjugate: in polyacrylamide gels, it stained with periodic acid-Schiff and less intensely with Coomassie brilliant blue; and in the analysis by RIA and double immunodiffusion, the antigenic activity of the preparation was markedly diminished by mild periodate oxidation.

Sera from 115 patients and normal donors were studied with the new RIA (Fig. 6). Of the

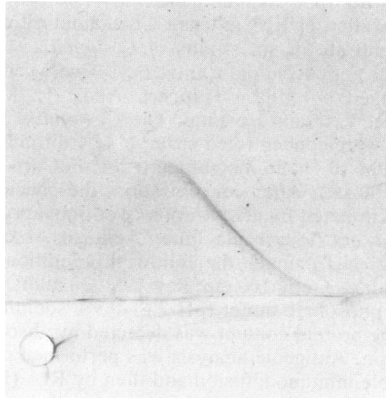

FIG. 4. Crossed immunoelectrophoresis of fraction 52 (see Fig. 1) run in the second dimension against anticoccidioidin reference sera. The well is at the cathode end of the plate.

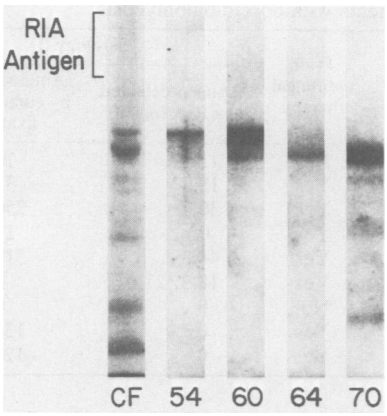

FIG. 5. Polyacrylamide gel electrophoresis of the coccidioidin culture filtrate (CF) and fractions 54, 60, 64, and 70 obtained from Sephacryl S300 gel chromatography. Migration was from top to bottom, and chromatography fractions are numbered by descending size. The *C. immitis* RIA antigen stained weakly with Coomassie brilliant blue and was not sharply resolved by polyacrylamide gel electrophoresis.

nine patients with active coccidioidomycosis (group I) antigenemia was detected in the sera of five. The mean antigen concentration in the positive serum samples was 25.8 ng/ml. Among the controls, antigenemia was not detected in one patient 4 days after surgical excision of a *C. immitis* coin lesion. In group II (Fig. 6), antigenemia was not detected in 4 of 5 patients with histoplasmosis or in 29 patients with other systemic mycoses, including systemic aspergillosis (12 patients), candidiasis (13 patients), zygomycosis (2 patients), and cryptococcosis (2 patients). In group III, antigenemia was not detected in 15 patients with bacterial sepsis, in 4 with mycobacterial, actinomycotic, or nocardial disease, or in 2 patients with viral hepatitis. In group IV, all of the serum samples from the 46 normal donors and 4 patients with malignancy were also antigen negative.

To better characterize the specificity of the *C. immitis* RIA, further analysis was performed with two other RIAs: one for candidal mannan, and one for an *A. fumigatus* carbohydrate. In group II, 13 patients had histologically documented invasive candidiasis. Antigenemia was detected by the candidal mannan RIA in 12 of them but fungal antigenemia was not detected with the *C. immitis* or *A. fumigatus* RIA. Similarly, 12 patients in group II had systemic aspergillosis. All were found to be antigenemic by an *A. fumigatus* antigen RIA, but the *C. immitis* and candidal RIA were negative.

The clinical and serological data for the patients with coccidioidomycosis are given in Table 1. Patient no. 1, an 83-year-old male, developed pulmonary symptoms after moving into the *C. immitis* endemic zone. Before therapy, *C. immitis* antigen was detected in serum samples at 15.9 ng/ml, and the titer for complement fixation antibody was 1:16.

Patient no. 2 was a 67-year-old male with a 5-year history of chronic progressive coccidioidomycosis of the lung. He had received 2.5 g of amphotericin B and had undergone a left pneumonectomy 1 year previously. When he relapsed, with weakness, weight loss, shortness of breath, and sputum cultures positive for *C. immitis*, he received another 1 g of amphotericin B and was referred to the University of Texas Health Science Center for further evaluation. Although the sputum, urine, bone marrow, and cerebrospinal fluid cultures taken there were negative for *C. immitis*, *C. immitis* antigen was found in serum samples at 18.7 ng/ml, and the complement fixation antibody titer was 1:32. The finding of antigenemia suggested a persistent infection despite the negative fungal culture. However, the microbiological study confirmed the serological findings when at follow-up 6 weeks later, a culture of the patient's sputum was again found to be positive.

In another case, a 20-year-old male developed massive cervical adenitis over a 12-month period. When evaluated, the patient had not responded to 4 months of antifungal chemotherapy and had persistently positive cultures for samples taken from multiple sinus drainage sites. *C. immitis* antigenemia was detected by RIA at a concentration of 30.7 ng/ml. After an additional 4 months of invasive amphotericin B chemotherapy, the patient clinically improved, the fistulas closed, fungal cultures became negative, and the antigenemia cleared, although complement fixation titers remained elevated. Howev-

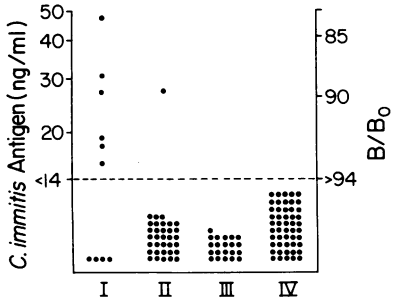

FIG. 6. *C. immitis* protein levels in sera. Groups: I, patients with coccidioidomycosis; II, control subjects with other systemic mycoses, III, patients with bacterial, mycobacterial, actinomycotic, or nocardial infections or with viral hepatitis; and IV, normal donors and patients with malignancy. B/B_o, Counts of radiolabeled antigen bound in standards or unknowns divided by counts bound in the absence of unlabeled antigen.

TABLE 1. Clinical and serological data for patients with coccidioidomycosis

Patient no.	Age (yr), sex	Type of coccidioidomycosis	Onset (mo before study)	Prior antifungal therapy	Serology	
					Antigen detected by RIA (ng/ml)	Antibody titer by complement fixation
1	83, M	Pulmonary	15	No	15.9	16
2	67, M	Chronic progressive pulmonary	60	Yes	18.7	32[a]
3	20, M	Chronic cervical lymphadenitis	12	Yes	30.7	256
4	12, M	Pulmonary	1	Yes	0	16[a]
5	38, F	Disseminated	12	Yes	44.2	512
6	40, M	Disseminated	1	Yes	18.3, 27.0	0[b]
7	47, M	Disseminated	1	No	0	0[b]
8	33, M	Disseminated	15	Yes	0	128
9	22, M	Meningitis	2	Yes	0	128

[a] Immunodiffusion complement fixation titer (11).
[b] Positive by immunodiffusion tube precipitin test (12) initially (patient 6) or in follow-up (patient 7).

er, 6 months later, while the patient was on less-intensive chemotherapy, a new fistula opened which was culture positive. Serum samples were not available to determine whether the antigenemia recurred with less-intensive chemotherapy before or at the time of relapse.

A 38-year-old female (patient 5) with disseminated coccidioidomycosis was treated with a total of 6 g of amphotericin B. When her serum was tested, *C. immitis* was detected at a concentration of 44.2 ng/ml, and the complement fixation titer was 1:512. The infection was resistant to therapy, and the patient later died.

A 40-year-old male (patient 6) developed fever and pulmonary infiltrates 5 weeks after receiving a cadaveric renal allograft and corticosteroid and cytotoxic chemotherapy. An open lung biopsy showed *C. immitis* spherules. Lung, bone marrow, and prostatic fluid cultures also grew *C. immitis*. Antibody serology by immunodiffusion complement fixation was negative, although an immunodiffusion tube precipitin assay was positive. At the time of biopsy, *C. immitis* antigenemia was initially detected at a concentration of 18.3 ng/ml but cleared with amphotericin B chemotherapy.

Antigenemia was not detected in the remaining three patients with extrapulmonary coccidioidomycosis. Complement fixation serologies were positive for two of these patients, and for the third, a renal transplant recipient, immunodiffusion tube precipitin antibody developed later while the patient was on antifungal chemotherapy. Sera for antigen testing were obtained only after the initiation of antifungal chemotherapy for patients 8 and 9. However, these cases were misclassified by the *C. immitis* RIA and thus were false-negative for the antigen serology. Of note, however, was the more severe illness of the two patients with antigenemia and extrapulmonary coccidioidomycosis. The protracted morbidity in patient 3 and the mortality of patient 5 were in contrast to patients 7, 8, and 9, in whom antigenemia was not detected; the latter patients had generally better and more rapid responses to therapy.

DISCUSSION

The utility of fungal antigen detecton in the diagnosis of opportunistic mycoses was shown in earlier studies (18–21). This study was undertaken to determine whether a similar diagnostic approach was feasible and of potential value for cases of coccidioidomycosis. I sought a serological test that did not depend on host immune response but rather on the detection of a *C. immitis* antigen(s) which might correlate with disease activity. To this end, serum samples from patients with coccidioidomycosis were examined for antigenic activity to several chromatography preparations. Antigenic activity from one high-molecular-weight coccidioidin preparation was detected in the serum samples. This preparation was further studied in light of the promising initial result and to develop a sensitive RIA. The high-molecular-weight pool was fractionated by gel exclusion chromatography. The purified antigen used for the RIA had the characteristics of a glycoprotein with a polymeric structure: it was heat stable, stained by periodic acid-Schiff and less intensely by Coomassie brilliant blue, and antigenically destroyed by mild periodate oxidation. Also, sharp resolution of the antigen was not achieved by polyacrylamide gel electrophoresis, isoelectric focusing, or chromatography over Sephacryl S300 or Sepharose 4B gels. This RIA antigen may be similar to or the same one responsible for the tube precipitin reactivity of coccidioidin described by Smith et al. (17).

In serum standards the RIA was sensitive to 14 ng of coccidoidin protein per ml. Preliminary acid-heat extraction increased the sensitivity of

the assay and permitted the detection of fungal antigen in clinical specimens as well as in immune complexes formed in vitro. These observations were similar to those made in cases of systemic candidiasis (19, 21) and aspergillosis (18, 20), in which fungal antigens appeared bound in serum complexes. To detect immune complexes in coccidioidomycosis, Yoshinoya et al. (23) recently evaluated serum samples from patients and controls with a Clq-binding assay, a Clq solid-phase assay, a monoclonal rheumatoid factor inhibition assay, and a monoclonal rheumatoid factor solid-phase assay. Increased concentrations of immune complexes were detected in 16 of 22 patients with active coccidioidomycosis by at least one of these four immunoassays, although 7 of 54 healthy controls also had positive tests.

In the present study, the *C. immitis* antigen RIA was evaluated with serum samples from 115 patients and normal donors. Anti-*C. immitis* antibodies detected by complement fixation or precipitins were found at the initial evaluation or the follow-up in the nine patients with active coccidioidomycosis. Antigenemia was detected in two of three patients with active pulmonary coccidiodomycosis, in a treated patient with persistent *C. immitis* lymphadenitis who had received drug treatment, in the one patient who died of the three patients treated for disseminated coccidioidomycosis, and in one of two untreated immunosuppressed patients with disseminated coccidioidomycosis. Antigenemia was not detected in 1 patient after the removal of a *C. immitis* coin lesion, in 4 with histoplasmosis, in 29 with other systemic mycoses, in 15 with bacterial sepsis, in 4 with mycobacterial, actinomycotic, or nocardial disease, or in 46 normal donors. However, a false-positive result was obtained from one patient with disseminated histoplasmosis of the five with pulmonary or disseminated histoplasmosis who were tested. The cross-reactivity of this *C. immitis* RIA antigen to histoplasmosis needs to be evaluated in a larger clinical group, although the drug therapy for coccidioidomycosis and histoplasmosis is similar and the areas in which these mycoses are endemic differ.

In a second study with the antigen RIA, a murine model of pulmonary coccidioidomycosis was investigated (M. H. Weiner, C. P. Andrews, M. Huppert, S. Norris, and S. Sun, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 484, 1982). In this experimental model, fungal antigen was detected in both sera and bronchoalveolar lavage fluids from *C. immitis* infected animals, but not in the body fluids from experimental control animals with *A. fumigatus* or *Klebsiella pneumoniae* pulmonary infections. This and other studies (1, 8) suggest that antigen immunodiagnosis of mycotic pulmonary infections may be improved by the combined analysis of sera and bronchoalveolar lavage fluids. Also, it may be that *C. immitis* antigen analysis of cerebrospinal fluid could be of immediate use, as capsular antigen assays are for cryptococcal meningitis.

In this retrospective study, *C. immitis* antigenemia indicated active or persistent disease. In chronic *C. immitis* infections, when antibody titers were elevated for prolonged periods, antigen detection augmented antibody serodiagnosis. Antigen serodiagnosis may also be of use in immunosuppressed patients with attenuated or delayed humoral responses and *C. immitis* infection. In the few cases in this study that were temporally well characterized, transient antigenemia (patient 6) and the absence of detectable antigenemia (patients 4, 7, and 9) were associated with a relatively benign clinical course marked by response to antifungal chemotherapy, surgical excision or drainage, or both. Before a correlation between antigenemia and prognosis can be made, however, more extensive serological experience needs to be gained in the longitudinal studies that are a part of the Coooperative Ketoconazole Treatment Project.

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