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Relationship of Progesterone- and Estradiol-Binding Proteins in *Coccidioides immitis* to Coccidioidal Dissemination in Pregnancy

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Pregnancy is a major risk factor for coccidioidal dissemination. Because rates of *Coccidioides immitis* growth and endospore release are stimulated in vitro by levels of unbound progesterone and 17β-estradiol that are achievable, in vivo, in the sera of pregnant women (i.e., 10^{-9} to 10^{-8} M), a specific-hormone-binding system in *C. immitis* was sought. Fungal cytosols were incubated with tritiated steroids plus or minus radioinert steroids to identify specific binding systems. All five strains of *C. immitis* tested exhibited specific saturable binding for progestin, estrogen, androgen, and (to a lesser extent) corticosterone and glucocorticoid hormone classes. Only low or inconsistent estrogen or androgen binding was found in *Blastomyces dermatitidis* and *Torulopsis glabrata*. *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*, and non-*albicans Candida* species showed no binding. Scatchard analysis of progestin and estrogen binding in *C. immitis* revealed a high-capacity, low-affinity binding system that was unaffected by RNase and DNase, but 40 to 60% degraded by trypsin or heating. Ammonium sulfate precipitation resolved a high-affinity, low-capacity binding system (K_d = 1.24×10^{-9} to 3.60×10^{-8} M; number of binding sites = 0.014 to 0.20 pmol/mg of protein). The K_d of this system is sufficient to compete for unbound hormone in the sera of pregnant women. The high-capacity, low-affinity system may serve as a repository for hormone before its attachment to the specific binder. These studies suggest that the effects of nanomolar concentrations of sex hormones on *C. immitis* may be mediated by a specific cytosol protein-binding system and that stimulatory events observed in vitro may have relevance for the mechanism of coccidioidal dissemination in pregnancy.

Coccidioidomycosis is a common infectious disease of the southwestern United States and areas of Mexico and Central and South America. Its etiological agent, *Coccidioides immitis*, is a biphasic fungus that is mycelial in its soil (saprobic) stage and characterized by a complex spherule-endospore cycle in its parasitic stage (23). Under ordinary circumstances, men are more likely than women to develop disseminated coccidioidal infection, the difference perhaps relating to greater opportunities for high-inoculum environmental exposures (5). If coccidioidomycosis is acquired during pregnancy, however, the risk of dissemination is increased directly as a function of the duration of the pregnancy (9).

The increased susceptibility of pregnant women to coccidioidal dissemination has been attributed to the relative immunosuppression that accompanies the gravid state (6). However, other infectious diseases show only a minor tendency to be aggravated by pregnancy, and we have sought additional explanations for the phenomenon. Recent studies from this laboratory have shown that the rate of growth of *C. immitis* in a defined medium and the subsequent rate of release of endospores are strongly stimulated by the presence of 17β-estradiol, progesterone, and testosterone (6). In contrast, 17α-estradiol (a relatively inactive stereoisomer of 17β-estradiol), cholesterol, ergosterol, and carbon-plus-nitrogen supplementation of the medium failed to reproduce these phenomena. Hormones with growth activity for *C. immitis* had no effect on diverse strains of *Cryptococcus neoformans*, *Candida* species, or *Petriellidium boydii*.

Since levels of 17β-estradiol and progesterone become markedly elevated as pregnancy progresses, and since the in vitro growth of *C. immitis* is stimulated by the concentrations of these hormones that occur in an unbound state in the serum of pregnant women, an in vivo acceleration of the parasitic cycle of *C. immitis*

appears likely. The resulting "population explosion" of endospores could be overwhelming in the presence of concomitant relative immunosuppression.

The purpose of this paper is to report the presence of specific progestin-, estrogen-, and androgen-binding activity in the cytosol of five strains of *C. immitis*. Corticosterone- and glucocorticoid-binding activity was demonstrable in lesser amounts. Representatives of five other fungal genera failed to demonstrate consistent sex hormone-binding activity. Hormone binders in *C. immitis* were generally of high capacity and low affinity for tritiated ligands. However, a high-affinity, low-capacity progestin binder was demonstrable in *C. immitis* cytosol as well. These binders may play a role in the stimulation by sex steroids of the growth rate of *C. immitis* and the consequent rate of endospore release.

MATERIALS AND METHODS

Fungi. Six fungal genera, including 15 separate strains, were examined for hormone-binding activity. All culture media were prescreened for hormone-binding capability and for the capacity to inhibit hormone binding in a standard assay, i.e., MCF-7 breast cancer cells (10). No anomalous effects could be attributed to the media. All studies were carried out under strict biohazard precautions, in a P3 facility (13, 24).

C. immitis. *C. immitis* C60 and C634 were recovered from cerebrospinal fluid; strains C566 and C665 were recovered from sputum. Strain C659 (Silveira) is a well-characterized laboratory strain that has been used in studies by numerous investigators (16).

Fungi were grown in the mycelial phase on glucose-yeast extract agar plates at room temperature for 6 to 8 weeks, as described elsewhere (23). A spinning bar technique was used to harvest arthroconidia (12). For the production of spherules, a heavy suspension of arthroconidia was inoculated into Converse liquid medium to a final concentration of 10^7 fungi per ml. After 48 to 96 h of incubation at 40°C (strain dependent), young spherules were harvested by centrifugation and washed in TESHMo buffer (10 mM Tris [pH 7.4] at 4°C, 1.5 mM EDTA, 0.25 M sucrose, 12 mM monothioglycerol, and 10 mM sodium molybdate).

Paracoccidioides brasiliensis. *P. brasiliensis* LA and C81, both clinical isolates, were grown in yeast form on Mueller-Hinton agar at 25°C for 11 days and then harvested into TESHMo buffer. The fungi were washed by centrifugation, enumerated with a hemacytometer, and evaluated for viability (≥95%) by methylene blue dye exclusion.

Other fungi. The following fungi were grown in yeast form in Sabouraud glucose broth at 37°C for 24 to 48 h, washed by centrifugation in TESHMo buffer, enumerated with a hemacytometer, and verified as ≥95% viable by methylene blue dye exclusion: *Blastomyces dermatitidis* 181-2 (a clinical isolate) and ATCC strains 18187 and 18188 (mating types of *Ajellomyces dermatitidis*); *C. neoformans* strains A and B (clinical isolates); *Candida tropicalis* strain S (clinical isolate); *Candida pseudotropicalis* strain S (clinical isolate); and *Torulopsis glabrata* VAR (clinical isolate) and ATCC 45545.

MCF-7 human breast cancer cell line. The MCF-7 cell line, derived originally from a patient with metastatic breast cancer (22), was employed as a positive control for hormone receptor activity. Cells (5×10^6) were plated in plastic roller bottles (850-cm^2 surface area) and grown in air at 37°C in a thermostatically controlled warm room. The growth medium consisted of Earle autoclavable minimal essential medium supplemented with 1% nonessential amino acids (GIBCO Laboratories, Grand Island, N.Y.), 2 mM L-glutamine (GIBCO), 0.006 μg of insulin per ml (Sigma Chemical Co., St. Louis, Mo.), and 25 μg of gentamicin per ml, all in 10 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; pH 7.4) containing 0.3% $NaHCO_3$ (7). The medium also contained 5% calf serum (GIBCO) that had been stripped of endogenous steroid hormones with a dextran-coated charcoal (DCC) pellet. A DCC suspension was prepared by mixing 0.25% Norit A (Sigma) and 0.0025% dextran (clinical grade; molecular weight, 79,000; Sigma) in 0.01 M Tris-hydrochloride buffer, with the pH adjusted to 8.0 at 4°C. A 25-ml amount of this suspension was centrifuged at 2,000 × *g* for 10 min, the supernatant was removed, and the pellet was suspended in 50 ml of serum. After a 30-min incubation at 45°C, the charcoal was removed by centrifugation, and the serum was stored at −70°C until used. The medium in the roller bottles was changed every 48 h.

Confluent MCF-7 cells were removed by a 5- to 10-min incubation at 37°C with 1 mM EDTA in Ca^{2+}-Mg^{2+}-free Earle balanced salt solution. Cells were then washed at 4°C, once with Earle solution and once in TEDMo buffer (10 mM Tris [pH 7.4] at 4°C, 1 mM EDTA, 1 mM dithiothreitol, and 10 mM sodium molybdate) with centrifugation.

Cell disruption. Because of potential biohazard dangers posed by the transfer of intact radiolabeled *C. immitis* from the P3 facilities where experiments were conducted to the common resource radioactivity counting room, all studies were conducted with the cytosols of disrupted fungi. To ensure that the methods of fungal disruption were not destructive to hormone-binding loci, MCF-7 breast cancer cells were employed as a positive control. MCF-7 cells contain cytosol receptors for hormones of the progestin, estrogen, androgen, corticosterone, and glucocorticoid classes (10).

Fungi. Fungal cell pellets (3 ml or ca. 10^{10} cells) were suspended in 10 ml of TESHMo buffer at 4°C and then combined with 20 ml of acid-washed beads (0.40- to 0.45-μm diameter) in a Duran flask (VWR Scientific, San Francisco, Calif.). The flask was placed in a Braun homogenizer (Melsungen, West Germany) that had been precooled in liquid CO_2, and cells were disrupted at low speed (2,000 rpm) with three 30-s bursts separated by 1-min cooling intervals. Microscopic examination indicated that fungal disruption was >99% complete. All subsequent procedures were carried out at 0 to 4°C. After the glass beads had settled, the supernatant was pipetted into a 50-ml conical tube, and the beads and cell pellet were washed with 5-ml portions of buffer. The combined supernatant and cell washings were centrifuged at 1,500 × *g* for 10 min (Beckman TJ6; Beckman Instruments, Inc., Cedar Grove, N.J.) to remove cell debris and any remaining

TABLE 1. Quantitative specific binding of tritiated ligands to cytosols of various fungi

Receptor class	Specific binding (pmol/mg of protein) to cytosols of:		
	C. immitis (5/10)[a]	*B. dermatitidis* (3/3)	*T. glabrata* (2/2)
Progestin			
R5020/R5020[b]	2.104 ± 1.194	0	0
Progesterone/progesterone	3.193 ± 1.529	0	0
Estrogen			
Estradiol/estradiol	1.695 ± 1.037	0.123 ± 0.214	0.173 ± 0.325
Estradiol/diethylstilbestrol	0.01 ± 0.028	Not done	Not done
Androgen			
R1881/R1881	1.447 ± 1.008	0.019 ± 0.32	0
Dihydrotestosterone/dihydrotestosterone	1.573 ± 1.172	0	0
Corticosterone			
Corticosterone/corticosterone	0.381 ± 0.238	0	0
Glucocorticoid			
Triamcinolone acetonide/triamcinolone acetonide	0.235 ± 0.396	0	0

[a] Number of strains/total experiments (duplicate).
[b] ^{3}H-ligand/cold competitor.

intact cells. The resulting supernatant was then centrifuged at 100,000 × *g* at 4°C for 1 h in a Beckman L5-50 ultracentrifuge. Protein concentrations in these cytosols were measured by the method of Bradford (1).

MCF-7 cells. The MCF-7 cell pellet was suspended in TEDMo buffer (1 ml/ml of pelleted cells) and divided into two equal portions. One portion was disrupted in a Braun homogenizer under the conditions described above for fungi. The other half was disrupted in a less traumatic manner in a hand-held Teflon-glass Dounce homogenizer (Kontes Scientific Glassware, Vineland, N.J.) by 35 strokes of the B pestle, the usual method for preparing MCF-7 cells for receptor assay studies (11). (Preliminary studies established that this method was inadequate to disrupt *C. immitis* or other fungi.) Both homogenates were centrifuged at 100,000 × *g* at 4°C for 1 h; protein concentrations were measured as noted above.

Ligands (steroids). The following ligands were used to assay for particular receptor classes: (i) Progestin—promegestone (17,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione [17α-*methyl*-^{3}H]) (R5020), 70 to 87 Ci/mmol, and unlabeled R5020; or progesterone [1,2,6,7-^{3}H(N)], 90 to 115 Ci/mmol, and unlabeled progesterone; (ii) estrogen—17β-estradiol [2,4,6,7-^{3}H(N)], 90 to 115 Ci/mmol, and unlabeled diethylstilbesterol or unlabeled 17β-estradiol; (iii) androgen—methyltrienolone (17β-hydroxy-17α-methylestra-4,9,11-trien-3-one [17α-*methyl*-^{3}H]) (R1881), 70 to 87 Ci/mmol, and unlabeled R1881 or dihydrotestosterone [1,2,4,5,6,7-^{3}H], 110 to 150 Ci/mmol, and unlabeled dihydrotestosterone; (iv) corticosterone—corticosterone [1,2,6,7-^{3}H(N)], 80 to 105 Ci/mmol, and unlabeled corticosterone; and (v) glucocorticoid—triamcinolone acetonide [6,7-^{3}H(N)], 30 to 50 Ci/mmol, and unlabeled triamcinolone acetonide.

Unlabeled R5020 and all tritiated steroids were purchased from New England Nuclear Corp. (Boston, Mass.). All other unlabeled steroids (including cholesterol and cortisol, which were used in competition studies) were purchased from Sigma.

Steroid receptor assay. To screen for the presence or absence of various steroid receptor classes, 200-μl samples of cytosol were incubated in duplicate with tritiated steroid (~2×10^{-8} M) plus or minus a 100-fold excess of the appropriate unlabeled steroid at 4°C for 3 h, a time that yielded maximum equilibrium binding without the deterioration of binding moieties. Binding was stable up to 24 h. Free ligand was separated from that which had been bound to cytosolic components by one of the following two methods.

(i) DCC method. The DCC method utilizes the well-known ability of DCC to adsorb steroid compounds that are free in solution (3). DCC (0.5 ml) was added to each tube containing labeled steroid, the tubes were shaken at high speed for 15 min (4°C) in a reciprocating shaker, and the free steroid-DCC complex was removed by centrifugation at 2,000 × *g* for 10 min. The amount of bound ligand was determined by counting 500-μl portions of the supernatant in 5 ml of toluene-based scintillation cocktail—4.0 g of PPO (2,5-diphenyloxazole), 0.05 g of POPOP {*p*-bis-[2]-(5-phenyloxazolyl)benzene; Amersham Corp., Arlington Heights, Ill.), and 1 liter of toluene—with a Beckman LS 230 counter at 38% efficiency.

(ii) HAP method. Hydroxylapatite (HAP; DNA-grade Bio-Gel HTP; Bio-Rad Laboratories, Richmond, Calif.) was washed several times with Tris-phosphate buffer (50 mM Tris and 10 mM potassium phosphate, monobasic [pH 7.2] at 4°C) to achieve pH 7.2 in the supernatant wash. The final packed HAP-to-buffer ratio was approximately 0.7. Cytosol samples were precipitated with 250 μl of this HAP slurry. Incubation was continued for 30 min at 4°C with gentle periodic vortexing. The tubes were centrifuged at 800 × *g* for 3 min. After the aspiration of the supernatants, the pellets were washed with 2 ml of TESHMo buffer containing 1% Tween 80 (Sigma), vortexed, and centrifuged. The supernatants were discarded. This procedure was repeated, and the final HAP pellets containing bound tritiated ligand were then suspended in 2 ml of ethanol and allowed to stand overnight at room temperature. The tubes were then gently vortexed and centrifuged at 800 × *g* for 10 min. The supernatant ethanol containing the extracted tritiated ligand was carefully decanted into counting vials and counted in the above cocktail at 25% efficiency.

In tubes containing tritiated ligand without any

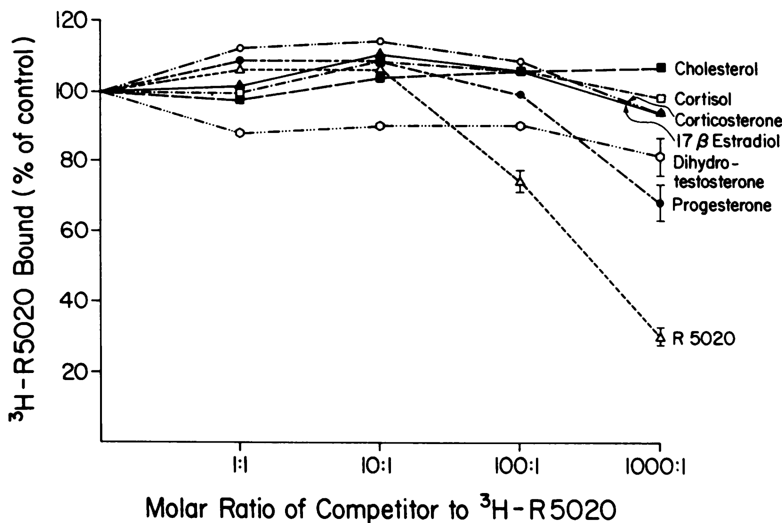

FIG. 1. Specificity of the *C. immitis* R5020 cytosol binder. Cytosol of strain C634 was incubated with 1×10^{-8} M ^{3}H-labeled R5020 for 3 h at 4°C plus or minus steroid competitors at the molar ratios indicated. Control (100%) binding of ^{3}H-labeled R5020 in the absence of competitors was 8.925 ± 0.65 pmol/mg of cytosol protein. Standard deviations ($n = 4$) are shown only for those data that differ significantly from 100% control binding and are provided only to indicate the mean and central tendency since statistical analysis employed nonparametric methods. The P value in each case = 0.014 (Mann-Whitney U test).

unlabeled steroid, the value derived represents total binding, i.e., both the specific binding of ligand to its receptor and nonspecific ligand binding to other components in the system. In tubes containing tritiated ligand and unlabeled steroid, since specific binding sites were saturated by unlabeled ligand that had been added in excess, whereas nonspecific binding sites were nonsaturable and available for labeling with tritiated ligand, the amount of tritiated ligand that was bound represents that portion that was attached to nonspecific binding sites. Accordingly, specific binding may be calculated from the formula total binding − nonspecific binding = specific binding.

Effect of enzymes on ligand binding. In some experiments the effects of RNase and DNase (Calbiochem-Behring, La Jolla, Calif.) and papain, chymotrypsin, and trypsin (Sigma) on ligand binding were examined. Enzymes were utilized in final concentrations of 100 μg/ml and incubated with cytosol at 37°C for 60 min.

Ammonium sulfate extraction of cytosol. Solid $(NH_4)_2SO_4$ (Ultrapure; Schwarz/Mann, Spring Valley, N.Y.) was added rapidly, with stirring, to a given volume of *C. immitis* cytosol until the desired percent saturation was achieved. Stirring was continued for a total of 30 min. After centrifugation at $20,000 \times g$ for 15 min, the supernatant was carefully removed, and the proteinaceous pellet was redissolved in TESHMo buffer. All operations were carried out at 0 to 4°C. The redissolved precipitate was immediately assayed for receptor activity.

Endogenous ligand. After the ultracentrifugation of *C. immitis* C634 cytosol at $100,000 \times g$ (1 h, 4°C), a lipid layer was apparent at the miniscus. This layer was carefully removed and dissolved by the addition of 500 μl of absolute ethanol. Portions (25 μl) of this material and of absolute ethanol were assessed for their ability to inhibit ^{3}H-labeled R5020 binding in the progestin receptor assay of crude cytosol.

Calculations. Hormone-receptor binding results were analyzed by the method of Scatchard (21). The dissociation constant (K_d) was estimated by the formula $K_d = -1/\text{slope}$. Total binding sites (n) were estimated from the Scatchard plot where n = the extrapolated x intercept of the straight line.

RESULTS

Table 1 summarizes the results of a single-point screening assay in which tritiated ligand at a concentration of $\sim 2.0 \times 10^{-8}$ M was exposed to cytosol in the presence or absence of unlabeled competitors. R5020- and 17β-estradiol-binding values in MCF-7 cells disrupted by a hand-held homogenizer or by the Braun homogenizer were virtually identical (data not shown), indicating that the latter method of disruption had no untoward effect on the detection of hormone binding. All five receptor classes were demonstrable in *C. immitis* cytosol, with progestin, estrogen, and androgen binding being dominant. Diethylstilbestrol was not an effective competitor for labeled 17β-estradiol, unlike the situation with mammalian cells (3). However, unlabeled 17β-estradiol itself was an excellent competitor, and with its use, specific 17β-estradiol binding was demonstrated. Estrogen or androgen binding was also demonstrable in *B. dermatitidis* and *T. glabrata* cytosols, but the values were inconsistent and highly variable. *C.*

neoformans and *P. brasiliensis* (two strains each) and *C. tropicalis* and *C. pseudotropicalis* (one strain each) showed no specific binding activity with any of the ligands tested.

Because progestin binding was not encountered with the other fungi and because the binding of progestin was comparatively greater than that with other hormone classes, extensive studies were carried out with the progestin-binding system. Preliminary experiments (^{3}H-labeled R5020 plus radioinert progesterone; [^{3}H]progesterone plus radioinert R5020) established that these ligands were identifying the same binding system. Accordingly, R5020 was utilized in subsequent experiments because of its high affinity and specificity for progesterone receptor and the greater stability of the R5020-progesterone receptor complex in mammalian systems (20).

Figure 1 shows the results of detailed competition studies aimed at defining more clearly the specificity of the R5020 binder. Strain C634 cytosol was used in these experiments. Specific binding for R5020 was 3.51 pmol/mg of protein. We found no competition by unlabeled cholesterol, cortisol, corticosterone, or 17β-estradiol, even at concentrations of 10,000:1 (data not shown). Unlabeled dihydrotestosterone showed slight, but significant, competition at a ratio of 1,000:1. Both R5020 and progesterone competed for ^{3}H-labeled R5020-binding sites under conditions suggesting the presence of a low-affinity, high-capacity binding system. Further experiments have shown that this inhibition was competitive for both progesterone and dihydrotestosterone (Lineweaver-Burk analysis at fixed concentrations of the inhibitor).

Saturation analysis was carried out with C634 cytosol to resolve the specific binding component and its saturability (Fig. 2A). There was considerable nonspecific binding (nonsaturable) in this cytosol, but a specific component was identifiable as well. With higher concentrations of ^{3}H-labeled R5020 (>100 pmol/ml), no further specific binding was encountered, indicating that saturation was complete. When subjected to Scatchard analysis (Fig. 2B), the data disclosed an apparently complex binding system. We considered that the possible degradation of binder at low steroid concentrations might also be a possible explanation, but rejected this on the basis of earlier experiments which showed binding to be unchanged after 24 h at 4°C. The treatment of cytosol with RNase, DNase, chymotrypsin, or papain had no effect on ^{3}H-labeled R5020 binding. However, trypsin or heating (60°C, 30 min) destroyed 40 to 60% of the binding activity. Thus, the binder was at least partially protein in nature.

To rule out the possibility of peculiarities unique to strain C634, studies were repeated with the cytosol of strain C60. In addition, because of the curvilinear nature of the C634 Scatchard plot, consideration was given to the possibility that the DCC separation of free from bound ligand might be introducing artifactual components to the curve at extremely high (incomplete adsorption of free ligand) or low (underestimation of free ligand) concentrations of ^{3}H-labeled R5020. Accordingly, the results of the DCC and HAP separation methods were compared.

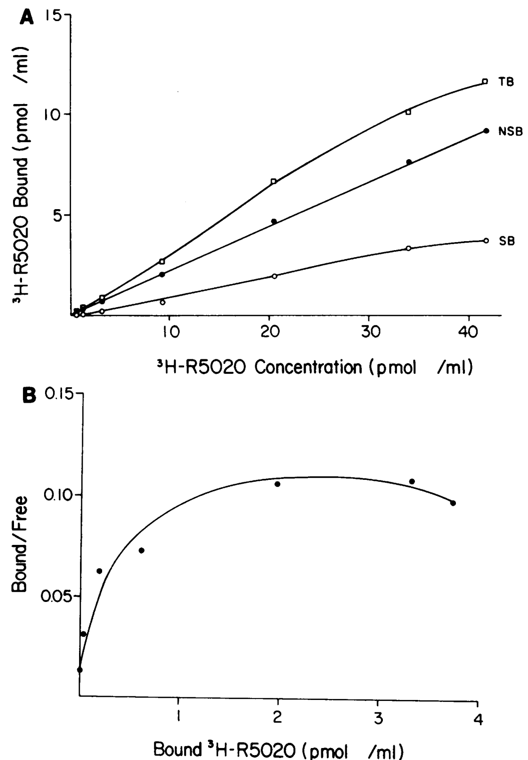

FIG. 2. (A) Saturation analysis of ^{3}H-labeled R5020 binding in *C. immitis* C634 cytosol. Free ligand was extracted by DCC. TB, Total binding; NSB, nonspecific binding; SB, specific binding. (B) Scatchard analysis of data in (A) showing apparent complex binding kinetics. Bound, Specifically bound ^{3}H-labeled R5020; free, free ^{3}H-labeled R5020.

The results of Scatchard analyses with strain C60 cytosol (and other strains [data not shown]) were qualitatively the same as those seen with strain C634 whether the DCC method (Fig. 3A) or the HAP method (Fig. 3B) was employed. All three figures show a great deal of scatter, and no obvious straight line can be deduced from these data. Although the amount of ^{3}H-labeled R5020 binding was substantially reduced by HAP (presumably by the deletion of non-protein-binding

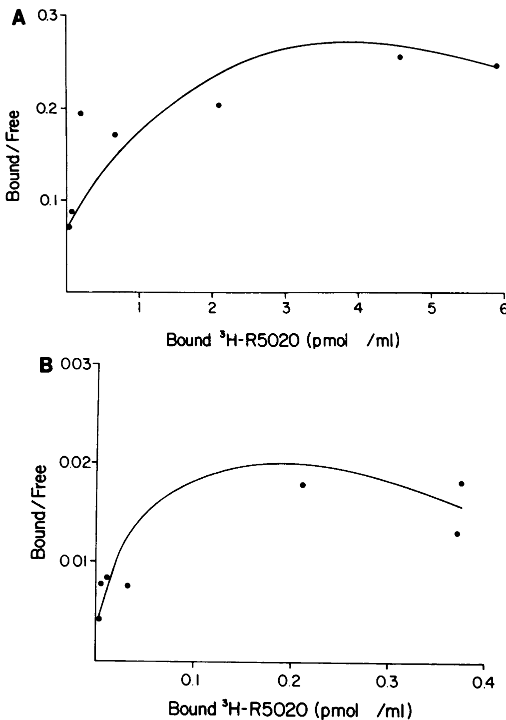

FIG. 3. (A) Scatchard analysis of DCC-extracted strain C60 cytosol. Bound, Specifically bound ^{3}H-labeled R5020; free, free ^{3}H-labeled R5020. (B) Scatchard analysis of HAP-extracted strain C60 cytosol. Bound, Specifically bound ^{3}H-labeled R5020; free, free ^{3}H-labeled R5020.

moieties), there was no alteration in the general shape of the curve. Similar curves were generated when 17β-[^{3}H]estradiol data were subjected to Scatchard analysis.

To determine whether a high-affinity progestin binder might be present in the protein fraction of *C. immitis* cytosol, but simply masked by the previously demonstrated high-capacity, low-affinity binder, an analysis of the progestin-binding characteristics of ammonium sulfate-precipitable cytosol fractions was undertaken. With the 80% ammonium sulfate precipitate, there were no changes in the overall shapes of the Scatchard plots. However, a grouping of data points at the very low concentration range of the plots suggested the presence of a high-affinity, low-capacity binding system. To analyze this binder, we confined Scatchard analyses to the low concentration range, and the number of data points was increased. Figure 4 shows a representative Scatchard analysis, and the pertinent kinetic parameters derived from such plots in three *C. immitis* strains are summarized in Table 2. We consistently demonstrated a low-capacity, high-affinity binding system for R5020 under these circumstances. The sigmoidal distribution of the data about a central inflection point was confirmed by plotting the moles of bound ligand against the log of the concentration of free ligand (15). Both the K_d and the number of binding sites are compatible with those encountered in mammalian systems. It should be emphasized that these values represent only approximations of the true parameters. A similar evaluation of the 17β-estradiol-binding moiety is in progress.

Preliminary analysis of the ethanol-extracted lipid supernatant of *C. immitis* cytosol revealed that this material inhibited R5020 binding to crude cytosol by a factor of 60%. Absolute ethanol used as the control inhibited binding by only 20%. These data seem to suggest that *C. immitis* itself produces a ligand that can compete with a recognized ligand (R5020) for the progestin binder. We have not as yet confirmed whether this inhibition is competitive in nature. Further analysis of this potential endogenous ligand is in progress.

DISCUSSION

In recently published experiments, we have shown that the rate of growth of *C. immitis* in vitro and the consequent rate of endospore release can be accelerated markedly by the addition of selected steroid sex hormones to the culture medium (6). Growth stimulation is directly related to the hormone concentration and occurs well within the range of the concentrations of unbound progesterone and 17β-estradiol that are encountered in the serum of pregnant women (i.e., 1 to 10% of total circulating hormone or approximately 10^{-9} to 10^{-8} M). Although testosterone is also stimulatory for *C. immitis* growth, serum concentrations do not reach a similar range under physiological conditions. We have speculated that the unusual susceptibility of pregnant women to coccidioidal dissemination may be related to the direct hormonal stimulation of fungal growth, with the result that the rate of endospore release outstrips the ability of the slightly impaired cell-mediated immune system of the pregnant female to contain the infection.

The stimulation of *C. immitis* growth by nanomolar concentrations of steroids suggests strongly that a specific recognition system must exist within the fungus for the detection of a "signal" that is present in such low molar concentrations. Accordingly, the present experiments were directed toward identifying specific sex hormone-binding activity in *C. immitis*. Because *C. immitis* poses an extreme biohazard (13, 24), we were forced to conduct our studies with fungal cytosols to avoid contaminating the common resource radionuclide counting facili-

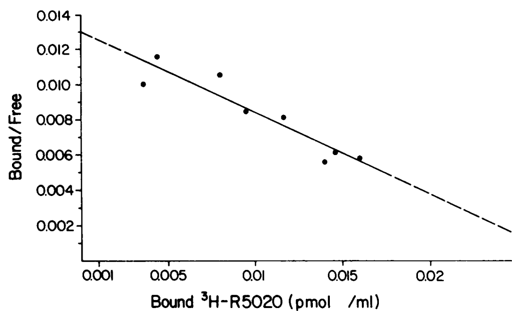

FIG. 4. Scatchard analysis of the 80% ammonium sulfate fraction of C634 cytosol. The analysis was confined to the low-concentration range (K_d = 2.16 × 10^{-9} M; n = 0.034 pmol/mg of protein). Bound, Specifically bound ^{3}H-labeled R5020; free, free ^{3}H-labeled R5020.

TABLE 2. Kinetic parameters derived from Scatchard analysis of the low-concentration range of 80% ammonium sulfate-precipitated *C. immitis* cytosol

Strain	K_d (M)	No. of binding sites (n) (pmol/mg of protein)
C634	4.60×10^{-9}	0.026
	2.16×10^{-9}	0.034
	1.04×10^{-8}	0.034
	3.60×10^{-8}	0.086
C566	1.10×10^{-8}	0.20
C659	1.34×10^{-8}	0.014
	1.24×10^{-9}	0.088

ties. This approach may have had the potential for introducing unwanted experimental variables into the test system, but cytosols are commonly used in mammalian hormone experiments (2), and particular precautions were taken to assure that the methods of fungal cell disruption were compatible with the maintenance of hormone-binding activity in a mammalian cell (MCF-7) control system.

Our studies have demonstrated the presence of progestin-, estrogen-, androgen-, corticosterone-, and glucocorticoid-binding activity in *C. immitis* cytosol and in the cytosol of MCF-7 tumor cells. No other fungi tested demonstrated this diversity of hormone-binding activity, nor did the few fungi that showed binding exhibit levels approaching those encountered with *C. immitis*.

Progestin-, estrogen-, and androgen-binding levels in *C. immitis* cytosol were both higher and more consistently elevated than those for corticosterone and glucocorticoid and were therefore accorded particular attention. Among the fungi tested, progestin (R5020, progesterone)-binding was unique to *C. immitis*, and therefore most studies were directed to analyzing this binding system. Although *Candida albicans* also contains a cytosol binder for progesterone (18, 19a), this binder shares specificity with corticosterone and has been considered to represent a corticosteroid receptor-like protein (18).

The R5020-binding system of *C. immitis* was highly specific, showing competition only with R5020 itself, progesterone, and dihydrotestosterone. The ability of dihydrotestosterone to compete for R5020-binding sites at higher molar concentrations is compatible with its known ability to compete under similar circumstances in mammalian systems (10). Although *C. immitis* cytosols demonstrated saturable specific binding for R5020 and for 17β-estradiol, the Scatchard plots showed a curvilinear data distribution that did not lend itself to easy analysis in terms of the calculation of the K_d or the number of binding sites. The complexity remained whether DCC or HAP was used to separate free and bound ligand. Thus, although *C. immitis* possessed specific binding activity for R5020 (progesterone) and 17β-estradiol, the binding was apparently of high capacity and only low affinity.

Earlier studies with *C. albicans*, using identical fungal disruption and hormone-binding methodology, had shown that the corticosterone binding system of this fungus was of high affinity and low capacity (K_ds ranged from 4.96 × 10^{-9} to 4.58 × 10^{-8} M and maximum members of hormone-binding sites ranged from 3.7 to 1,450 fmol/mg of protein [19a]). Moreover, Scatchard analysis of saturation data with *C. albicans* (and MCF-7 cell) cytosol had shown none of the complexity that was subsequently encountered in the *C. immitis* system. It was for this reason that studies were pursued that ultimately demonstrated a high-affinity, low-capacity progestin binder in *C. immitis*.

Despite our demonstration of a binder with a K_d sufficient to compete for unbound progesterone in the serum of pregnant women, we are not prepared to discount the potential significance of the much larger quantity of high-capacity, low-affinity binding material. Perhaps, as has been shown in some mammalian systems (4), the low-affinity binder serves as a repository for hormone within the fungus, whereas the high-affinity binder is more directly involved in growth stimulation.

Our studies to date have demonstrated the growth stimulation of *C. immitis* by nanomolar concentrations of selected sex steroids and the presence of specific binders for these hormones in *C. immitis* cytosol. Whether the mechanism of fungal stimulation is based on a classical cytoplasm-to-nucleus transfer of bound ligand remains to be ascertained. Until the system is further understood, we believe that the material

in the cytosol must be considered a hormone binder, rather than a classical receptor.

Several recent studies now suggest that steroid hormone receptors and, indeed, steroid sex hormones themselves may be represented earlier in phylogeny than has been previously suspected (18). A putative estrogen receptor system has now been described for *Saccharomyces cerevisiae* (8), and the corticosterone receptor system in *C. albicans* has been further analyzed (17). In both of these systems, the term receptor was used by the investigators, even though the biological events, if any, that result from fungus-hormone interaction were not defined. To date, the relationship of these systems to fungal pathogenesis has only been speculative.

The demonstration of hormone binders in *C. immitis* and the demonstrated stimulation of fungal growth by physiologically meaningful levels of sex hormones, levels that are routinely achieved in mid- to late pregnancy, suggest that there may be a relationship between hormone binders and coccidioidal pathogenesis. Interaction between fungal hormones and mammalian cells is well documented. For example, β-resorcylic acid lactones produced by *Fusarium* species fit mammalian estrogen receptors and stimulate biochemical events normally associated with 17β-estradiol (14, 19). The result of this interaction is the appearance of female traits in swine that ingest *Fusarium* sp.-contaminated feed. Our studies suggest that the converse situation may also obtain. That is, elevated sex steroid levels in pregnant women may stimulate biochemical events in *C. immitis* that are expressed as an increased rate of fungal growth. Studies to clarify these biochemical events are in progress.

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