115

INFECTION AND IMMUNITY, Nov. 1984, p. 346–353
0019-9567/84/110346-08$02.00/0
Copyright © 1984, American Society for Microbiology
Vol. 46, No. 2

# Estrogens Inhibit Mycelium-to-Yeast Transformation in the Fungus *Paracoccidioides brasiliensis*: Implications for Resistance of Females to Paracoccidioidomycosis

ANGELA RESTREPO,[1] MARIA E. SALAZAR,[1] LUZ E. CANO,[1] E. PRICE STOVER,[2] DAVID FELDMAN,[2] AND DAVID A. STEVENS[3,4]*

*Laboratory of Mycology, Corporacion de Investigaciones Biologicas, Hospital Pablo Tobon Uribe, Medellin, Colombia[1]; Division of Endocrinology, Department of Medicine, Stanford University Medical School, Stanford, California 94305[2]; Division of Infectious Diseases, Santa Clara Valley Medical Center and Stanford University Medical School,[3] and the Institute for Medical Research,[4] San Jose, California 95128*

Received 30 May 1984/Accepted 24 July 1984

**Evidence that disease due to the thermally dimorphic fungus *Paracoccidioides brasiliensis* occurs post-puberty predominantly in males led us to hypothesize that hormonal factors critically affect its pathogenesis. We show here that estrogens inhibit mycelial- to yeast-form transformation of *P. brasiliensis* in vitro. Transformation of three isolates was inhibited to 71, 33, and 19% of the control values in the presence of $10^{-10}$, $10^{-8}$, and $10^{-6}$ M 17β-estradiol, respectively. The synthetic estrogen diethylstilbestrol was active but less potent than estradiol, whereas testosterone, 17α-estradiol, tamoxifen, and corticosterone were inactive. This function was specifically inhibited, since yeast-to-mycelium transformation, yeast growth, and yeast reproduction by budding were unaffected by 17β-estradiol. Of note is the fact that mycelium-to-yeast transformation occurs as the first step in vivo in the establishment of infection. The cytosol of the three isolates studied possesses a steroid-binding protein which has high affinity for 17β-estradiol. We believe that this binding protein represents a *P. brasiliensis* hormone receptor which can also recognize mammalian estrogens. We hypothesize that the ability of estrogen to decrease or delay mycelium-to-yeast transformation at the initial site of infection contributes to or is responsible for the marked resistance of females, and that the binder described is the molecular site of action.**

Paracoccidioidomycosis, which is caused by the dimorphic fungus *Paracoccidioides brasiliensis*, is endemic in regions of Latin America (25). A striking fact in its epidemiology is the increased frequency of the disease in males; the male/female ratio of the disease in Colombia is 48:1 (22). In contrast, skin test studies in endemic areas indicate that infection is equally common in males and females, because there is no sex-based difference in reactors to the fungal extract, paracoccidioidin (1, 13, 27). It is also noteworthy that there is no sex-based difference in those who acquire the disease before puberty (23).

These data suggest that hormonal factors play a critical role in the pathogenesis of the disease. Although increased male susceptibility occurs in a variety of infectious diseases and sex hormones influence immune responses and killing by toxic oxygen metabolites (11, 14), such findings would not appear to sufficiently explain why the sex ratio in paracoccidioidomycosis is so much more pronounced than in other infections. We therefore hypothesized that sex hormones might directly affect the behavior of the fungus in vivo. It has been shown that fungi use message molecules (some of these are steroidal) to modulate certain functions (3, 9, 12), analogous to hormone systems in higher eucaryotic organisms. More recently, it has been shown that fungi pathogenic for humans may be influenced by mammalian steroid hormones: a protein in *Candida albicans* which binds corticosterone and progesterone with high affinity and specificity has been characterized (18), and *Coccidioides immitis* growth has reportedly been accelerated by mammalian steroid hormones (reviewed in reference 6).

In our initial studies with *P. brasiliensis*, we demonstrated a high-affinity, low-capacity, estrogen-binding protein in cytosol from the yeast form (19). In this report we show that estrogens, but not other steroid hormones, suppress in vitro the transition between the mycelial and yeast forms of *P. brasiliensis*. This transition must occur during the initial establishment of infection after the fungus gains access to the host through its portal of infection, the lungs (28). This effect, moreover, appears specific for this *P. brasiliensis* function in our studies. We also show that the isolates affected contain a protein which binds estrogens. This binding protein is believed to be the molecular site of action of the hormonal effect described.

## MATERIALS AND METHODS

**Isolates.** Isolates of *P. brasiliensis* (Mon, Gir, and Ru) from patients were maintained by monthly transfers in the mycelial or yeast form on agar slants prepared with modified McVeigh-Morton (MVM) medium (26) at 20 to 25 or 36°C, respectively.

**Effect of hormones on mycelium-to-yeast transformation.** Mycelia were harvested from cultures grown for 6 to 10 days at 20 to 25°C in liquid MVM medium on a gyratory shaker (120 rpm). Homogenization was performed in a laboratory blender in one or two 5-s bursts until the supernatant, when observed in a microscope after 10 min of gravity sedimentation, consisted of well-dispersed hyphal fragments. This supernatant was adjusted to a turbidity equivalent to tube 5 of the McFarland scale (8). A microculture system previous-

* Corresponding author.

ly described (24) was used for the assays. The compounds to be assayed were diluted in ethanol and mixed with agitation at 56°C with MVM medium and agar or agarose to a final concentration of $2 \times 10^{-6}$ M. Tenfold or 100-fold dilutions of these compounds in medium plus agar were also prepared. Controls consisted of identical concentrations of ethanol in medium plus agar (0.6%, vol/vol) for the $2 \times 10^{-6}$ M preparation and 10- or 100-fold dilutions which matched the dilutions of the hormone in ethanol. Ethanol at these concentrations was later shown not to affect transformation compared with the controls lacking ethanol. The agar mixtures were allowed to gel at 4°C, and 1-$cm^2$ blocks were transferred aseptically to a sterile microscope slide. Inoculum (0.005 ml) was applied to the blocks and allowed to penetrate for 5 min. A sterile cover slip was then applied and sealed with a 1:1 mixture of petrolatum and paraffin, and the slide was incubated in a humid chamber at 36°C for 5 days. Two microcultures were set up at each concentration in each experiment for each isolate studied, and at least three experiments per isolate were performed. The blocks were examined microscopically (100 cells per microculture), and the percentage of mycelial fragments untransformed or transformed to yeast-form cells (28) was found. Data were expressed as percent transformation compared with the corresponding ethanol (or no ethanol) control. In a few experiments, the controls transformed at ≤30%; these results were not analyzed.

**Effect of hormones on yeast-to-mycelium transformation.** Yeast-to-mycelial-form transformation was studied by a method similar to that described above. The inoculum was a suspension of yeast-form cells grown for 3 days in liquid MVM medium at 36°C on a gyratory shaker (200 rpm). The cells were centrifuged ($60 \times g$) to remove clumps and leave only single yeast cells in the supernatant for use in the assay. The suspension was adjusted to a turbidity equivalent to tube 3 of the McFarland scale. After inoculation of the agar as stated above, the incubation step was performed at 25°C for 3 days. For the microscopic examination, yeast cells which had transformed into well-developed hyphae were scored. Transformation in the controls was 79 to 90%.

**Chemicals for binding studies.** 17β-[6,7-$^3$H]estradiol (47 Ci $mmol^{-1}$) was obtained from Amersham Corp., Arlington Heights, Ill. Nonradioactive steroids were purchased from Steraloids, Wilton, N.H. All chemicals were purchased from Sigma Chemical Co., St. Louis, Mo., unless otherwise noted.

**Preparation of cytosol.** Stock cultures of the clinical isolates were maintained on agar at 35°C. These yeast-phase cells were inoculated by loops into modified MVM liquid medium and grown at 35°C (19). Contamination was excluded by subculturing on blood agar plates before harvesting. Cells were harvested, washed, and suspended in Tris-molybdenum homogenization medium (pH 7.8) (18). The cells were lysed by vigorous agitation with 250- to 300-μm glass beads on a vortex mixer (17). The lysate was centrifuged in a microfuge (Beckman Instruments, Inc., Fullerton, Calif.) at $9{,}000 \times g$ for 15 s, and cytosol was prepared from the supernatant by ultracentrifugation at 4°C as previously described (18).

**Binding studies.** Cytosol was incubated with tritiated estradiol for 3 h at 0°C, a period sufficient for the mixture to reach equilibrium (17). Nonspecific binding was assessed in all experiments by incubating identical samples with a 500-fold molar excess of unlabeled estradiol. Bound steroid was separated from free steroid on a microgel exclusion column (18). The column was precentrifuged at $130 \times g$ for 2 to 3 min at 0 to 4°C to remove most of the buffer from the column. Cytosol samples (200 μl) were loaded onto the microcolumn, which was placed in a 13-mm tube and centrifuged, and the eluate, containing protein-bound hormone, was collected. The cytosol protein concentration was measured by the Coomassie dye binding technique (2).

**Effect of hormone on yeast-form growth and budding.** Spectrophotometric analyses of growth and growth inhibition were performed with one isolate in the presence of either hormone or appropriate ethanol control by using techniques previously described (10). Cultures were inoculated with yeast-form cells at $10^3$ cells per ml from MVM agar slants and were incubated at 35°C in MVM medium on a gyratory shaker (250 rpm).

Liquid medium cultures were studied for the rate of bud formation in a similar fashion, with a starting inoculum of $10^5$ $ml^{-1}$. Cultures were set up in duplicate, 200 cells from each pair of cultures were examined, and the number of buds per cell was counted.

## RESULTS

**Effect of mammalian hormones on conversion of form in *P. brasiliensis*: mycelium-to-yeast conversion.** Three isolates were studied extensively. In the absence of added hormones, mycelium-to-yeast transformation occurred in 35 to 80% of cells in these experiments (Fig. 1). We found that of five hormones, analogs, and antagonists examined in detail, 17β-estradiol consistently inhibited mycelial- to yeast-form transformation in vitro over the range of concentrations tested (Fig. 2). This was the case with all three isolates, although there were small differences between isolates. For the three isolates we found that 17β-estradiol inhibited transformation at 64 to 77, 24 to 46, and 12 to 26% of the control value at concentrations of $2 \times 10^{-10}$, $2 \times 10^{-8}$, and $2 \times 10^{-6}$ M, respectively (Fig. 2). *P. brasiliensis* Gir was the least susceptible at the lower concentrations.

There was also a clear dose-response correlation with each of the three isolates for the three 17β-estradiol concentrations. Such correlation was seen with only one other agent, the synthetic estrogen diethylstilbestrol (DES). In seven of the nine data sets (Fig. 2), 17β-estradiol was the most inhibitory agent tested. In the other two sets (*P. brasiliensis* Ru, $2 \times 10^{-6}$ and $2 \times 10^{-8}$ M), DES was slightly more inhibitory. The specificity of the effect is shown by the lack of influence of 17α-estradiol (Fig. 2), a closely related molecule which has no or weak estrogen effect in mammals. A few experiments were also done with 10-fold dilutions of agents instead of 100-fold, and they showed similar evidence of a dose response with 17β-estradiol (data not shown). In addition to the hormones shown in Fig. 2, other experiments (data not shown) showed that corticosterone ($2 \times 10^{-6}$ to $2 \times 10^{-8}$ M) had no effect on transformation.

In summary, the mean of the data from three strains (Fig. 2) showed inhibition of mycelium-to-yeast transformation at 71, 33, and 19% of the control value with 17β-estradiol concentrations of $2 \times 10^{-10}$, $2 \times 10^{-8}$, and $2 \times 10^{-6}$ M, respectively. The DES effect at these concentrations resulted in inhibition at 85, 54, and 37% of the control transformation rates, respectively. None of the other three agents showed a significant effect; the mean for the three isolates ranged from 84 to 120% of controls for all three concentrations and was 106 to 120% for all three agents at $2 \times 10^{-10}$ and $2 \times 10^{-8}$ M.

Three to seven experiments were performed for each isolate with 17β-estradiol or DES. If all experiments were analyzed for statistical purposes, the mean percent of con-



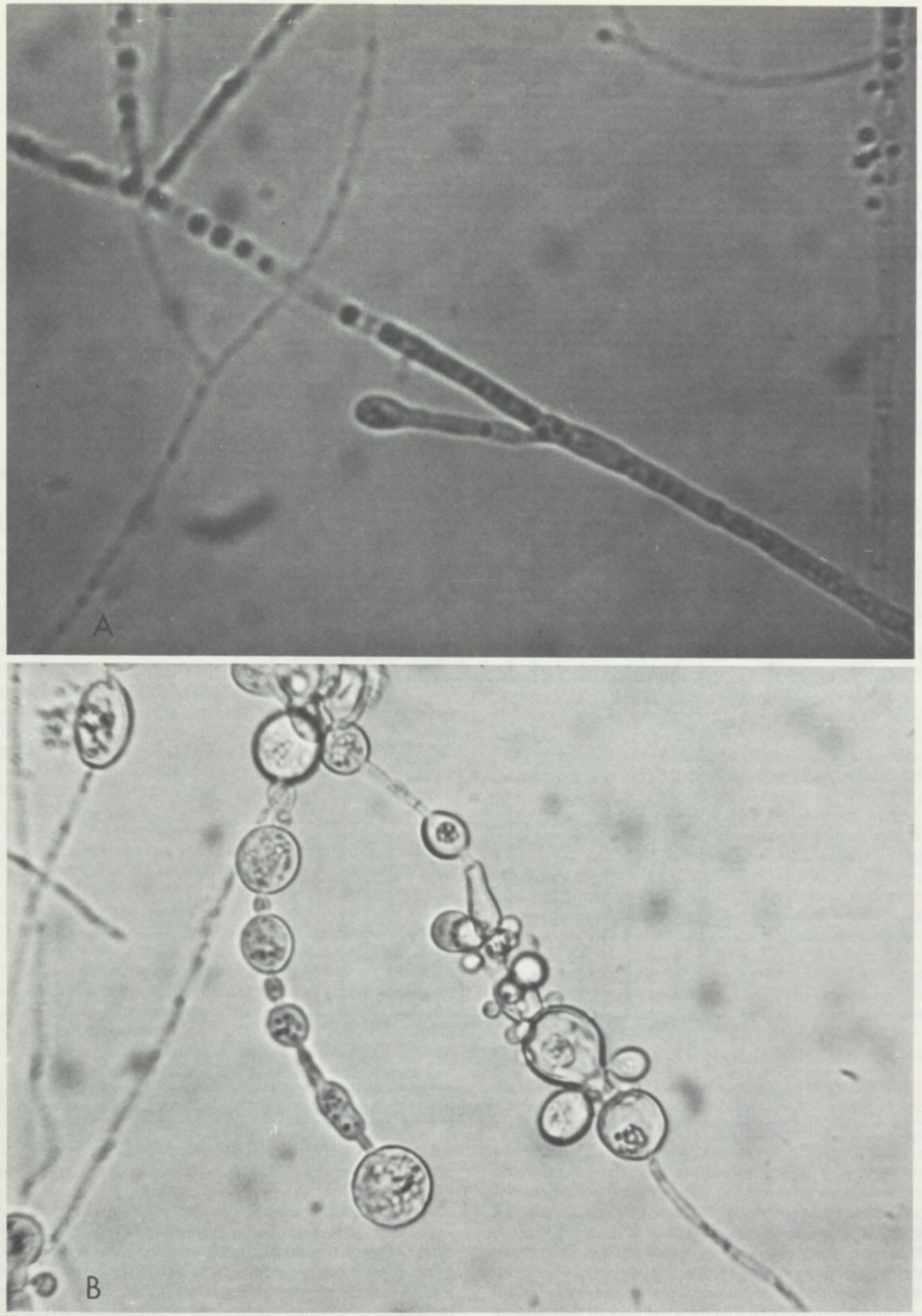


FIG. 1. Mycelium-to-yeast transformation in *P. brasiliensis*. (A) Mycelial fragments, none transformed. Appearance of the microcultures before incubation, as well as when transformation is blocked by hormone. (B) Transformation of mycelial fragments to the yeast form after incubation at 36°C for 5 days in agar microcultures. The initial step is formation of rounded structures which then develop into individual yeast cells, as can be noted in the figure. The yeast cells then reproduce by multiple budding, and some mother cells with several attached daughter cells can also be seen. For reference to all structures seen, the diameter of individual yeast cells is 5 to 15 μm.

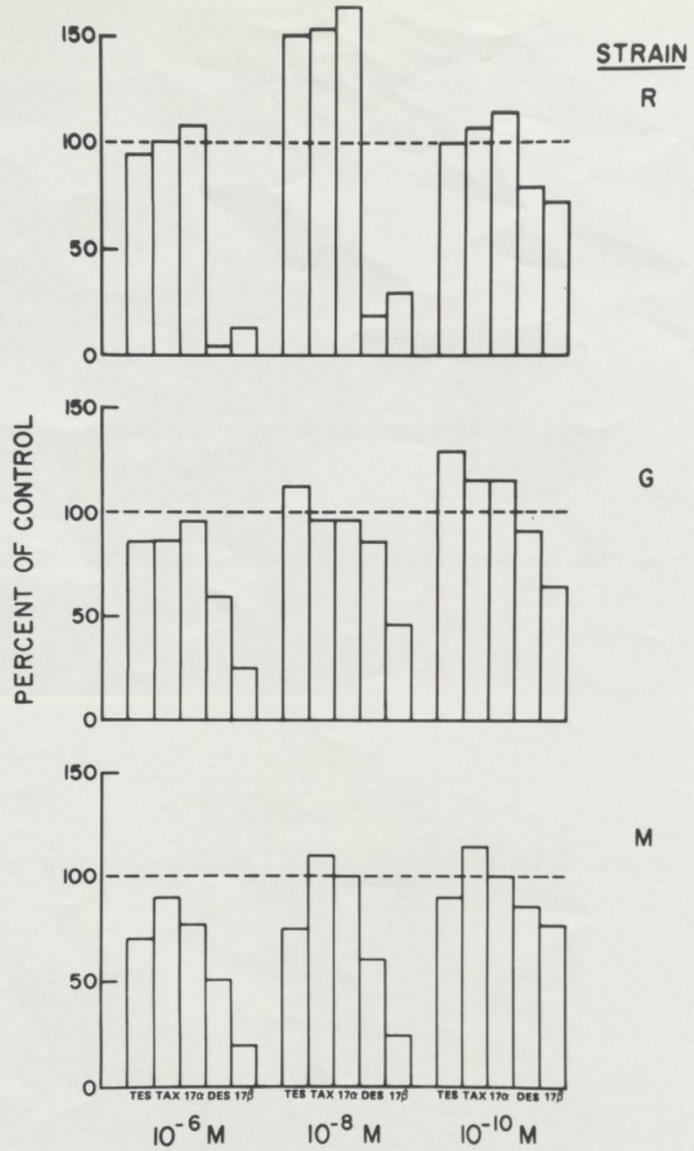


FIG. 2. Effect of five hormones, analogs, or antagonists at three concentrations on three clinical isolates of *P. brasiliensis*. Data are expressed as percent transformation compared with concurrent control (see text). The concentrations of agents tested were $2 \times 10^{-6}$, $2 \times 10^{-8}$, and $2 \times 10^{-10}$ M. Abbreviations: TES, testosterone; TAX, the estrogen antagonist tamoxifen; 17α, 17α-estradiol; 17β, 17β-estradiol; R, Ru; G, Gir; M, Mon.

trol ± standard error at $2 \times 10^{-10}$, $2 \times 10^{-8}$, and $2 \times 10^{-6}$ M for 17β-estradiol were 78.5 ± 5.1 (14 experiments), 31.7 ± 5.0 (20 experiments), and 11.0 ± 3.0 (20 experiments), respectively, all $P < 0.001$ by Student's $t$ test. For DES, these experimental means ± standard error were 89.5 ± 4.5 (11 experiments), 59.2 ± 9.5 (12 experiments), and 40.8 ± 7.2 (12 experiments), respectively, giving $P$ values of <0.05, <0.01 and <0.001, respectively.

**Further observations on mycelium-to-yeast conversion.** Some of the cells which transformed from the mycelial to the yeast form developed further in the latter form during the experimental interval, giving rise to multiple daughter yeast cells in the characteristic manner of this organism (Fig. 1). If the cells proceeding further in the yeast form were enumerated independently and the percentage of the control value was calculated, inhibition by 17β-estradiol was again noted. A representative set of data with *P. brasiliensis* Gir indicates transformation from mycelial cells to multiply budding yeast cells to be 33% compared with concurrent ethanol controls at $2 \times 10^{-10}$ M and 2% at $2 \times 10^{-6}$ M. These results are similar to those shown in Fig. 2, and reflect the fact that mycelium-to-yeast transformation must take place before multiply budding yeast cells can arise. Since, in the presence of 17β-estradiol, mycelial cells were markedly inhibited from transforming to yeast cells, the impairment of formation of multiply budding yeast cells in the experimental interval presumably reflects this. Experiments with each of the three strains were performed, with similar results. Further experiments (described below) directly addressed the question of hormonal effect on yeast cell budding.



We obtained information on the conservation, through multiple in vitro passages in the mycelial form, of the ability of *P. brasiliensis* to be inhibited by estradiol in its mycelium-to-yeast transformation. Several sets of the studies on transformation included above were performed within 3 months of isolation of the organism from the patients. Studies performed more than 1 year after isolation, after monthly serial passages, showed indistinguishable susceptibility to 17β-estradiol.

**Effect of hormones on conversion: yeast-to-mycelium conversion.** The specificity of the hormonal effect on mycelium-to-yeast transformation was also suggested by the studies with yeast-to-mycelium transformation. Only a slight effect was seen with any of the agents tested at any concentration on any of the three isolates (the results with the three were indistinguishable). At the highest (and nonphysiological) concentration, $2 \times 10^{-6}$ M, inhibition of transformation expressed as above as percent transformation (mean of three isolates) compared with the control values was 66% for DES, 79% for testosterone, 82% for tamoxifen, 86% for 17β-estradiol, and 95% for 17α-estradiol. At $2 \times 10^{-8}$ M, no agent resulted in transformation of <75% of the control values, and at $2 \times 10^{-10}$ M, none resulted in <89% of controls. These effects did not attain statistical significance in the series of experiments performed.

**Effect of estrogen on yeast-form growth.** Further studies were performed to determine the specificity of the function of 17β-estradiol on *P. brasiliensis*. The yeast form of one isolate was grown in the presence of 10-fold dilutions of 17β-estradiol in medium from $10^{-6}$ to $10^{-10}$ M. Spectrophotometric readings to determine growth were obtained 1, 2, 3, 6, and 8 days after initiation of the cultures. Cultures grown in tubes free of hormone or ethanol did not show significant growth (<99% transmission) above the threshold of detection of the instrument (ca. $4 \times 10^5$ yeast cells per ml) (10) until day 6. At that time, and on day 8, there was no difference between hormone-free, ethanol-free tubes and tubes containing hormone or the corresponding ethanol control. As an example (comparing means of pairs of tubes at each point), on day 8, when the percent transmission in hormone-free, ethanol-free tubes was 83%, the percent transmission in the presence of 10-fold dilutions of hormone beginning with $10^{-6}$ M was 86, 86, 84, 83, and 89%. In the corresponding ethanol controls the transmission was 86, 89, 89, 90, and 84%, respectively.

**Effect of estrogen on yeast-form budding.** Although there was no evidence of hormone effect on yeast growth as assayed spectrophotometrically in vitro (a measure of mass), another way that hormone could affect pathogenicity in vivo would be to affect the process of multiple budding. If greater numbers of viable (even if smaller) progeny were produced from a single mother cell in the presence of hormone, the infection would be expected to be more difficult for host defenses to contain. The data on cells which had transformed from the mycelial to the yeast form and then to multiply budding yeast cells are also relevant to this question.

The same isolate as in the preceding experiment and similar culture conditions, for 5 to 6 days, were used, and 10-fold dilutions of hormone from final concentrations of $10^{-6}$ to $10^{-9}$ M were studied with corresponding ethanol controls. As an example, on day 6 the percentage of cells showing 0, 1, 2 to 5, 6 to 9, 10 to 15, and >15 buds per cell in controls was 10, 18, 39, 15, 12, and 7, respectively, and at $10^{-7}$ M hormone it was 15, 26, 33, 9, 10, and 9. There was no evidence of significant hormone effect at any concentration.

**Analysis of [$^3$H]estradiol binding.** Details of the molecular characteristics of a *P. brasiliensis* cytosol protein which binds 17β-estradiol are presented elsewhere (19). Cytosol from each of the three isolates studied here was incubated with several concentrations of [$^3$H]estradiol between 6.5 and 130 nM. A representative isotherm and analysis (29) of the binding data from one experiment are shown in Fig. 3. The isotherm shows specific binding that is saturable at ca. 65 nM. A plateau of ca. 135 fmol of [$^3$H]estradiol bound per mg of protein was obtained. The data, plotted by the Scatchard method, yielded an apparent dissociation constant of 16 nM and a binding capacity of 153 fmol/mg of protein. The results of two Scatchard analyses for each isolate are given in Table 1. Each possesses a high-affinity, low-capacity binder for 17β-estradiol.

We obtained data on the conservation of the binder through multiple in vitro passages in the yeast phase. Indistinguishable 17β-estradiol binding was found in cytosol obtained from *P. brasiliensis* serially passaged for at least 24 months.

**Steroid specificity of the cytosol binder.** The specificity of the binding site for [$^3$H]estradiol was also examined. Of the compounds tested, 17β-estradiol was by far the most potent competitor for [$^3$H]estradiol binding in each of the three isolates (Fig. 4). Each of the other compounds tested showed only weak activity. 17α-estradiol, the relatively inactive stereoisomer, was ca. 2 to 3% as potent as 17β-estradiol. Both DES and testosterone were weak competitors, ca. 1% as potent as 17β-estradiol. The inhibition by DES, in contrast to the other compounds, was not dose responsive in two strains.

The binding data for these three isolates of *P. brasiliensis* are consistent with our initial results in one isolate (19). In that study it was also shown that the cytosol binder was protein in nature, with an apparent molecular weight of ca. 50,000, and that the [$^3$H]estradiol binding was reversible.

## DISCUSSION

We show in this report that estrogens inhibit mycelium-to-yeast transformation of *P. brasiliensis*. In contrast, yeast-to-mycelium transformation, yeast growth, and yeast budding are unaffected by hormones. The isolates possess a cytosolic binding protein which has high affinity and stereo specificity for estradiol. It is our hypothesis that this binding site is involved in the regulation of the function we demonstrated, and that in vivo, inhibition of transformation by estrogen in the lungs of females at the portal of infection provides an explanation for the resistance of females to the disease. Such inhibition could effectively reduce the propagation of the

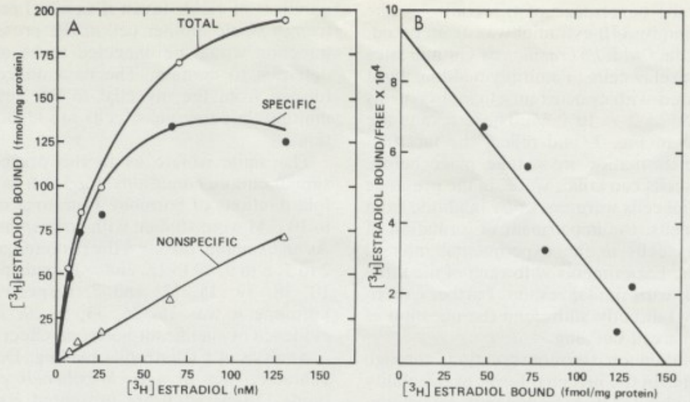


FIG. 3. Equilibrium analysis of [$^3$H]estradiol binding in *P. brasiliensis* Gir. Binding was studied for 3 h at 0°C. (A), Isotherm of total (○), specific (●), and nonspecific (△) binding at various concentrations of [$^3$H]estradiol. (B), Scatchard analysis of the specific binding taken from the isotherm. The $K_d$ in this study was 16 nM and the binding capacity was 153 fmol mg$^{-1}$ of cytosol protein.

initial inhaled inoculum, improving the capability of the host to prevent progression of infection, or a delay in transformation could allow females preferentially to develop an immune response. In the transformation studies, mycelial fragments were used rather than purified spores because of the difficulties in obtaining and working with spores; however, we believe that observations with mycelial fragments are relevant to observations about transformation from the mycelial phase and would also apply to spores. At such time that advances in technology enable similar studies with spores, this assumption can be tested directly.

Muchmore et al. (21) showed that both forms of *P. brasiliensis* were inhibited in their in vitro growth by the female sex hormones estradiol and progesterone, and that stilbestrol was even more potent than these. This finding is probably not relevant to our findings because the hormone concentrations used by Muchmore et al. ($1.5 \times 10^{-5}$ to $3 \times 10^{-5}$ M estradiol) were far above the physiological range (concentrations as high as $1 \times 10^{-9}$ to $2 \times 10^{-9}$ M are reached during the menstrual cycle) (30). Estradiol, even at $2 \times 10^{-10}$ M, showed an effect on function in our system, and it is possible that significant effects might be seen at even lower concentrations. The characteristics of the binding protein presented are consistent with an effect at physiological hormone concentrations. An exact concordance cannot, however, be expected because of unknown factors in the in vitro system which may affect hormone activity (e.g., interaction with medium components or agar, effect of microaerophilic environment, etc.). Other possible distortions of a precise concordance include the following: the affinity of hormone to binder in intact cells may be different from that in disrupted cells, other circulating estrogens and progesterone may occupy the estradiol site in vivo with an additive effect, the intact fungus may concentrate hormone, or a function may be regulated with only a few binding sites occupied.

TABLE 1. Scatchard analyses of [$^3$H]estradiol binding to cytosols from three clinical isolates of *P. brasiliensis*[a]

| Isolate | $K_d$ (nM)[b] | $N_{max}$ (fmol/mg of protein)[b] |
|---|---|---|
| Ru | 17 ± 2 | 116 ± 38 |
| Mon | 17 ± 8 | 303 ± 51 |
| Gir | 10 ± 6 | 194 ± 41 |

[a] Cytosol preparations from each isolate were incubated with different concentrations of [$^3$H]estradiol for 3 h at 0°C. Bound [$^3$H]estradiol was separated from free hormone on microcolumns. The apparent dissociation constant ($K_d$) and binding capacity ($N_{max}$) were determined by Scatchard analysis in each experiment.

[b] Each value represents the mean ± range for two experiments performed on each isolate.

Moreover, the characteristics of the hormone binding have been studied so far with the yeast form, and only in preliminary fashion as yet with the mycelial form, because of biohazard considerations with the latter (25). The amount of binder present in the mycelial form and its binding characteristics may be different and may explain disparities between hormone concentrations showing a functional effect and the saturation of the binder at that concentration. This disparity was most marked with DES. It is of interest that DES, an apparently weak competitor for [$^3$H]estradiol binding in *P. brasiliensis*, has a potency equal to that of 17β-estradiol in the mammalian receptor system (5). Many of the factors we have discussed which could affect the quantitative correlations are susceptible to further experimental study. These necessary further studies may provide data which support or reject our hypothesis.

We earlier presented (19) hormone binding studies with a single strain. We present here, with three isolates, data on the hormone effect on the transformation function and binding data from the same isolates. The characteristics of the binding by cytosol from these isolates were similar (Table 1), and were similar to those of the strain previously reported. Thus the presence of a binder is shown to be a property of many *P. brasiliensis* strains. Moreover, the variation in the hormone effect on transformation in the three isolates detailed here was also small (expressed above as ranges given for Fig. 2, and standard error for all experiments). If greater variations were found between binding characteristics and functional responses, this could argue against our hypothesis, or it might suggest that there are important components of the transformation response in addition to the binding protein. Other new findings of interest here include the demonstration of the specificity of the 17β-estradiol effect on *P. brasiliensis* functions in mycelium-to-yeast transformation, the lack of effect of 17α-estradiol and tamoxifen, and the conservation of the binder and of the 17β-estradiol susceptibility of mycelium-to-yeast transformation through serial in vitro passages.



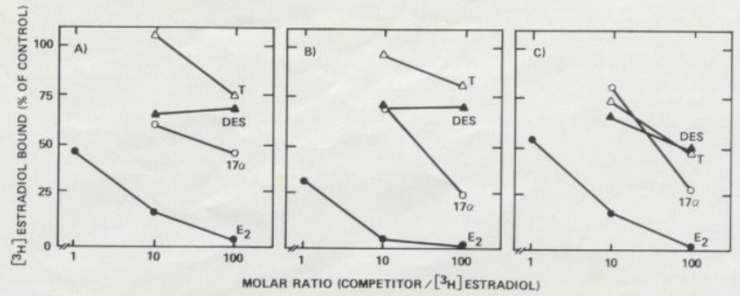


FIG. 4. Specificity of the *P. brasiliensis* binder by competition analysis. Cytosol was incubated with 130 nM [$^3$H]estradiol with and without the indicated concentration of competitor. The binding in the absence of competitor was taken as 100%. (A), (B), and (C), Results from *P. brasiliensis* Ru, Mon, and Gir, respectively. Each point represents the mean of 5 to 8 determinations. Abbreviations: $E_2$, 17β-estradiol; 17α, 17α-estradiol; T, testosterone.

The presence of a *P. brasiliensis* binding protein with high affinity for estrogen suggests conservation throughout evolutionary development of eucaryotes, a conservation in the fungus almost certainly unrelated to the property of binding vertebrate hormone. This finding suggests the existence of an endogenous fungal ligand with specificity for the binder and at least partial structural similarity to estrogen. It is of interest that an estradiol-binding protein has been demonstrated in *Saccharomyces cerevisiae* (7), a protein similar in density and binding specificity to that in *P. brasiliensis*. In *S. cerevisiae*, evidence for an endogenous ligand was presented. Morphological observations, such as the presence of Woronin bodies in the mycelial septa, indicate that *P. brasiliensis* is a member of the ascomycete fungi (4). One would then expect sexual (perfect) forms to exist, and by analogy with other fungi (3, 9, 12), the ligand-receptor system may be related to the mating processes. Recently, sexual (perfect) forms have been described for other dimorphic fungi pathogenic for man (15, 16, 20). The possibility of occupancy by the hypothesized endogenous ligand of the sites in *P. brasiliensis* which bind estradiol is another consideration in quantitatively comparing the data on effect on transformation and the binding data with circulating hormone concentrations in humans.


## ACKNOWLEDGMENTS

This work was supported by grants from the Fondo Colombiano de Investigaciones Cientificas (Colciencias), Colombia (grant no. 97145-3-06-80), Public Health Service grants no. AI 20409 and GM-28825 from the National Institutes of Health, and grants no. INT-80-18623 and INT-8302725 from the National Science Foundation.

We thank William Brown, Division of Biostatistics, Department of Family, Community and Preventive Medicine, Stanford University Medical School, for assistance with statistical analysis, Suzanne Smith and Joanne Brown for assistance with the manuscript, and Elmer Brummer for assistance with the experiments on budding.


## LITERATURE CITED


1. **Albornoz, M. B.** 1975. Resultado de las encuestas epidemiologicas realizadas con paracoccidioidina en Venezuela. Castellania **3**:37–40.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein dye binding. Anal. Biochem. **72**:248–254.
3. **Bu'Lock, J. D.** 1975. Hormones in fungi, p. 345–368. *In* D. R. Berry and J. E. Smith (ed), The filamentous fungi. John Wiley & Sons, Inc., New York.
4. **Carbonell, L. M., and J. Rodriguez.** 1968. Mycelial phase of *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis*: an electron microscope study. J. Bacteriol. **96**:533–543.
5. **Chen, T. L., and D. Feldman.** 1978. Distinction between alpha-fetoprotein and intracellular estrogen receptors: evidence against the presence of estradiol receptors in rat bones. Endocrinology **102**:236–244.
6. **Drutz, D. J., and M. Huppert.** 1983. Coccidioidomycosis: factors affecting the host-parasite interaction. J. Infect. Dis. **147**:372–390.
7. **Feldman, D., Y. Do, A. Burshell, P. Stathis, and D. S. Loose.** 1982. An estrogen binding protein and endogenous ligand in the yeast *Saccharomyces cerevisiae*: a possible steroid-receptor system. Science **218**:297–298.
8. **Finegold, S. M., W. J. Martin, and E. G. Scott (ed.).** 1978. Bailey and Scott's diagnostic microbiology, p. 488–489. C. V. Mosby Co., St. Louis, Mo.
9. **Flegel, T. W.** 1981. The pheromonal control of mating in yeasts and its phylogenetic implication: a review. Can. J. Microbiol. **27**:373–389.
10. **Galgiani, J. N., and D. A. Stevens.** 1976. Antimicrobial susceptibility testing of yeasts: a turbidimetric technique independent of inoculum size. Antimicrob. Agents Chemother. **10**:721–726.
11. **Goble, F. C., and E. A. Konopka.** 1973. Sex as a factor in infectious disease. Trans. N.Y. Acad. Sci. **35**:325–346.
12. **Gooday, G. W.** 1974. Fungal sex hormones. Annu. Rev. Biochem. **43**:35–49.
13. **Greer, D. L., D. D'Acosta, and L. Agredo.** 1974. Dermal sensitivity to paracoccidioidin and histoplasmin in family members of patients with paracoccidioidomycosis. J. Trop. Med. Hyg. **23**:87–98.
14. **Klebanoff, S. J.** 1979. Effect of estrogens on the myeloperoxidase-mediated antimicrobial system. Infect. Immun. **25**:153–156.
15. **Kwon-Chung, K. J.** 1972. Sexual stage of *Histoplasma capsulatum*. Science **175**:326.
16. **Kwon-Chung, K. J.** 1975. A new genus, *Filobasidiella*, the perfect state of *Cryptococcus neoformans*. Mycologia **67**:1197–1200.
17. **Loose, D. S., and D. Feldman.** 1981. Characterization of a unique corticosterone-binding protein in *Candida albicans*. J. Biol. Chem. **257**:4925–4930.
18. **Loose, D. S., D. A. Stevens, D. J. Schurman, and D. Feldman.** 1983. Distribution of a corticosteroid-binding protein in Candida and other fungal genera. J. Gen. Microbiol. **129**:2379–2385.
19. **Loose, D. S., E. P. Stover, A. Restrepo, D. A. Stevens, and D. Feldman.** 1983. Estradiol binds to a receptor-like cytosol binding protein and initiates a biological response in *Paracoccidioides brasiliensis*. Proc. Natl. Acad. Sci. U.S.A. **80**:7659–

7663.
20. **McDonough, E. S., and A. L. Lewis.** 1967. *Blastomyces dermatitidis*: production of the sexual stage. Science **156**:528–529.
21. **Muchmore, H. G., B. A. McKown, and J. A. Mohr.** 1972. Effect of steroid hormones on the growth of *Paracoccidioides brasiliensis*, p. 300–304. *In* Proceedings of the First Pan American Symposium on Paracoccidioidomycosis. Pan American Health Organization Scientific Publication no. 254, Washington, D.C.
22. **Restrepo, A.** 1978. Paracoccidioidomycosis: actualizacion. Acta Medica Colombiana **3**:33–66.
23. **Restrepo, A.** 1981. Paracoccidioidomycosis, p. 1500–1505. *In* R. D. Feigen and J. D. Cherry (ed.), Textbook of pediatric infectious disease. The W. B. Saunders Co., Philadelphia.
24. **Restrepo, A., L. E. Cano, C. de Bedout de G., E. Brummer, and D. A. Stevens.** 1982. Comparison of various techniques for determining viability of *Paracoccidioides brasiliensis* yeast-form cells. J. Clin. Microbiol. **16**:209–211.
25. **Restrepo, A., and D. L. Greer.** 1983. Paracoccidioidomycosis, p. 43–64. *In* A. F. DiSalvo (ed.), The occupational mycoses. Lea & Febiger, Philadelphia.
26. **Restrepo, A., and B. E. Jiménez.** 1980. Growth of *Paracoccidioides brasiliensis* yeast phase in a chemically defined culture medium. J. Clin. Microbiol. **12**:279–281.
27. **Restrepo, A., M. Rebledo, S. Ospina, M. Restrepo, and A. Correa.** 1968. Distribution of paracoccidioidin sensitivity in Colombia. Am. J. Trop. Med. Hyg. **17**:25–37.
28. **Rippon, J. W.** 1980. Dimorphism in pathogenic fungi. Crit. Rev. Microbiol. **8**:39–97.
29. **Scatchard, G.** 1949. The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. **51**:660–672.
30. **Williams, R. H. (ed.).** 1981. Textbook of endocrinology, 6th ed., p. 365. The W. B. Saunders Co., Philadelphia.