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Candida albicans morphogenesis is influenced by estrogen

S. White[a] and B. Larsen[b,*]

[a]Department of Obstetrics and Gynecology, Marshall University School of Medicine, Huntington (West Virginia 25703, USA)
[b]Department of Microbiology, Immunology and Molecular Genetics, Marshall University School of Medicine, Huntington (West Virginia 25703, USA)

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Abstract. Conversion of *Candida albicans* from yeast to mycelial growth is believed to be associated with the organism's virulence. We investigated the role of mammalian hormones in initiating this transformation. Three clinical isolates of *Candida albicans* were tested for their ability to produce germ tubes under various conditions. Controlled hormonal conditions were provided by stripping rabbit serum with activated charcoal. Steroid compounds under investigation were added back to the stripped serum and yeast were inoculated into the test materials. Microscopic counts of germinated versus ungerminated cells were used as an indicator of morphogenic transformation. The percent of yeast cells germinating was profoundly reduced in stripped compared to unstripped serum. The addition of 1 μM estradiol, cholesterol or testosterone only slightly increased levels of germination above that seen in controls. Estradiol at concentrations 100 times less, however, proved a strong inducer of germination. Cholesterol did not synergize germination when combined with estradiol and the alpha isomer of estradiol had almost no activity as an inducer of morphogenic change in *Candida albicans*. We conclude that beta estradiol was a morphogenic inducer in three clinical isolates of *Candida albicans* but only at concentrations typical in vivo.

Key words. *Candida*; yeast; estrogen; germination.

Candida albicans benignly colonizes the vaginal epithelium of women but causes symptoms only sporadically. The conditions within the microenvironment that change the relationship of this organism to the host from commensal to pathogenic have been a subject of conjecture for many years. Various microorganisms are known to increase their virulence in response to chemical signals such as temperature change, iron concentration or osmotic stimulus, but such environmental cues have not been identified for *Candida albicans* [1–4].

Clinical observation has indicated that pregnancy is a time characterized by an increased development of symptomatic yeast infection, and animal studies have demonstrated that vaginal colonization of rats can only occur after estrogen priming [5]. While this relationship of estrogen to infection or colonization may only reflect the hormonal effects on vaginal epithelium, it may also result from a direct effect of estrogen on the yeast. We have previously shown a tropic effect of estradiol on some strains of *Candida albicans* [6], and other investigators have determined that this organism possesses an estrogen-binding protein [4].

Estrogen seems an attractive candidate for an environmental signal that may upregulate the organism's virulence, since it is present inside the mammalian host but not in most environments outside the host. One potential virulence factor is the organism's morphogenic transformation from yeast to hyphal growth [4, 8, 9]. Thus, we have undertaken this study to examine the role of estrogen in directing the morphogenesis of *Candida albicans*.

* Corresponding author. Current address: Dean for University Research, University of Osteopathic Medicine and Health Sciences, 3200 Grand Avenue, Des Moines (Iowa 50312, USA), Fax +1 515 271 1714, e-mail: blarsen@uomhs.edu

Materials and methods

Three isolates of *Candida albicans* were randomly selected from our culture collection of clinical isolates. Verification of the effect of serum stripping was done using a traceable laboratory strain, ATCC 10231, which was handled identically as clinical isolates. Clinical strains were initially identified by formation of typical brown colonies on BIGGY agar (composition as g/L: bismuth ammonium citrate 5 g, sodium sulphite 3 g, glucose 10 g, glycine 10 g, yeast extract 1 g, agar 16 g) and by microscopic morphology and germ tube formation in normal human serum. These organisms were maintained on Sabouraud's dextrose agar (SDA). Prior to use they were regrown for 24 hours on SDA and isolated colonies picked for suspension in test materials described below.

Serum used in this study was normal rabbit serum obtained by cardiac puncture from three New Zealand white rabbits. The blood was allowed to clot and the blood was centrifuged at $600 \times g$ for 20 minutes. Serum was removed and pooled and stored at -20 °C until needed. Stripped serum was prepared by adding 10% activated charcoal (Sigma Chemical Co., St. Louis, MO) and incubating at 37 °C for 1 hour with periodic mixing. The charcoal-treated serum was centrifuged at room temperature for 10 minutes at $10{,}000 \times g$ and used as described below. An aliquot of the same serum pool was reserved as an unstripped control.

Serum additives included beta estradiol (1,3,5[10]-estratiene-3,17 β-diol), alpha estradiol (3,17α-dihydroxy-1,3,5[10]-estratriene), cholesterol (5-cholesten-3β-ol) and testosterone (4-androsten-17β-ol-3-one), each purchased from Sigma Chemical Co., St. Louis, MO. Stock solutions of 1×10^{-4} M were prepared in absolute ethanol, stored at -20 °C and further diluted in alcohol as needed. When these steroid compounds were added to serum, the amount added did not exceed 1% of the total of the total volume. Controls consisted of absolute ethanol added to serum in a final concentration of 1%.

Evaluation of germ tube formation was accomplished by suspending fresh colonial growth from a SDA plate in duplicate into microtitre wells containing 200 µl of serum or stripped serum with additives as noted above. The yeast suspension was incubated at 37 °C for up to 240 minutes. Aliquots were removed from the wells at various times and examined microscopically. Outgrowth from the surface of a yeast cell was only considered to be a germ tube if the structure had developed sufficiently to identify an elongated structure with parallel sides, so as not to count buds (blastospores) as germ tubes. At least 100 cells from each well were counted to establish the percent of cells that had germinated and results were reported as the average of duplicate determinations.

Identical aliquots of yeast strain ATCC 10231 inoculated into six wells containing rabbit serum or six wells of charcoal-stripped serum showed reproducible levels of germination for replicate determinations. The organism produced $29.8\% \pm 3.3\%$ (S.D.) germination at 105 minutes in unstripped serum and $25.7\% \pm 2.9\%$ (S.D.) at 240 minutes in stripped serum. Under both conditions the relative standard deviation was approximately 11%, implying that differences in germination greater than 10–15% are likely to represent valid differences.

Results

Germination of *Candida albicans* occurs in serum from humans or animals and is one of the tests used to identify the organism. The promotion of yeast to mycelial transition may be regulated by many factors, but we are particularly interested in the possibility that mammalian hormones may play a role in this process. We investigated whether normal rabbit serum stripped of its steroid hormones could support germination of *Candida albicans*. Figure 1 shows the microscopic appearance of a single strain (strain 49) of *Candida albicans* incubated with unstripped or stripped rabbit serum. A clear difference was noted, with stripped serum having apparently lost its ability to promote germination. It was clear from the microscopic appearance that the absence of germination was not the result of the yeast dying in the presence of the stripped serum since many buds indicating continuing replication were seen.

To follow up on this observation, we made a detailed evaluation of the time course of germ tube formation of three strains of yeast in stripped and unstripped normal rabbit serum at 45 minute intervals. As shown in figure 2, the three strains varied in their apparent intrinsic propensity to develop germ tubes, but nevertheless, the stripped serum was clearly different with respect to its ability to support germination. The ability to produce germ tubes was not eliminated but only delayed, suggesting a factor or factors present in the normal serum promoted more rapid initiation of germination. These results were also confirmed with ATCC 10231 which showed a delay in germ tube formation of approximately 135 minutes in stripped serum compared to unstripped serum and after 240 minutes incubation, 26% of ATCC 10231 cells had germinated in stripped serum compared to 100% germination in unstripped serum.

Activated charcoal binds a variety of compounts including steroids, suggesting that the addition of these

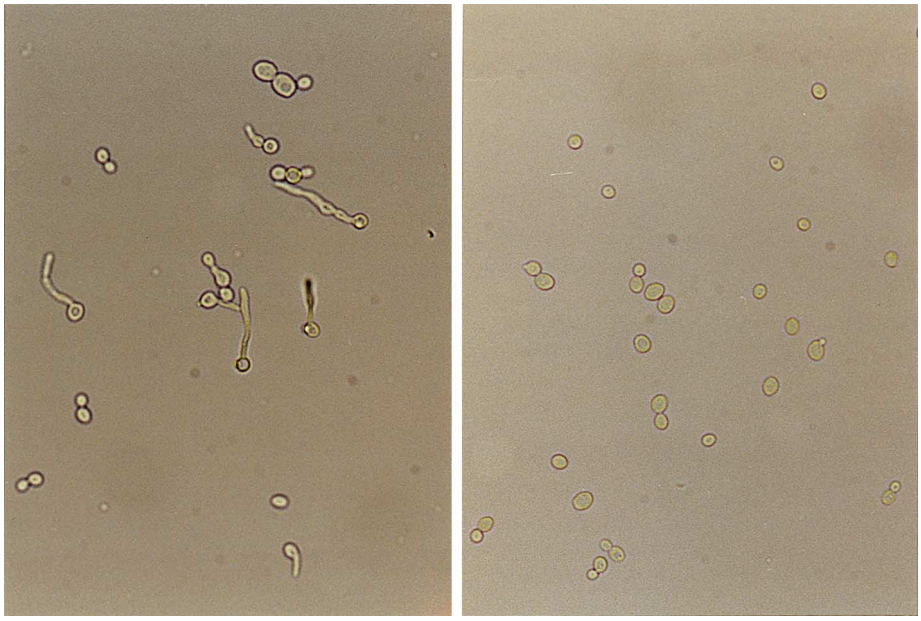

Figure 1. Loss of *Candida albicans* germination in charcoal stripped normal rabbit serum. A heavy suspension of yeast was made in normal rabbit serum or serum that had been treated with activated charcoal. The yeast suspensions were incubated for 180 minutes and photographed (original magnification, 400×). Yeast in unstripped serum appears in the left panel and the identical strain of yeast in stripped serum is seen in the right panel.

compounds to stripped serum may restore germination. We supplemented stripped serum with beta estradiol, cholesterol or testosterone at a concentration of 1 μM/L. This concentration is greater than normal serum levels, but we selected these higher concentrations with the expectation that if an effect of steroid existed, it would be most clearly evident at a hyperphysiological concentration. As shown in figure 3, cholesterol and estradiol increased germination of strain 49 slightly above control (stripped serum without steroid added) levels. This effect on germination was small but definite, although it suggested that factors other than steroids may be primarily responsible for the germination-promoting effect of whole serum. Alternatively, the hyperphysiological concentration of the test compounds might have been responsible for the poor rate of germ tube formation.

To determine if lower concentrations of steroid could promote germ tube formation, we tested concentrations of estradiol down to 1×10^{-8} M. Figure 4 shows that lower concentrations of estradiol produced higher levels of germination than were seen at the μM level. Because the rate of germ tube formation was a variable characteristic that was highly dependent on the age of the starter culture and perhaps other ill-defined factors, we performed additional experiments with estradiol concentrations as low as 1×10^{-10} M to confirm the effect of low levels of estradiol on germination and found that germination was consistently increased above that seen in stripped serum alone (data not shown). However, even the highest rates of germination did not equal that seen in unstripped serum.

We tested a combination of estrogen and cholesterol each at 1×10^{-9} M in an attempt to determine if the two steroid compounds would act synergistically to promote germ tube formation. As shown in table 1, there was no apparent synergy between these compounds. Indeed, cholesterol may actually antagonize the effect of estradiol.

Finally, we determined if *Candida albicans* responded to the non-estrogenic alpha isomer of estradiol to the same extent it did to the more estrogenic beta isomer. Stripped rabbit serum was supplemented with the beta and alpha forms of estradiol at a concentration of 1×10^{-9} M and as shown in figure 5, the alpha isomer had virtually no germination-promoting activity.

Discussion

One of the most powerful concepts to develop from the contemporary understanding of host-microbe interactions at the molecular level is that of global regulation of virulence [1–3]. While adaptation to the host has long been considered a part of the virulence panoply of microorganisms, only recently have microbial systems been identified that show the existence of genetic master switches that sense some environmental cue and generate an intracellular signal that activates a diverse collec-

tion of genes that adapt the microorganism to its altered environment. The role of global gene regulation in virulence appears to allow a microorganism to move from saprophytic growth to pathogenicity, or if in a pathogenic state move from one tissue site in the infected host to another, as in the case of spread of infecting organisms from mucosa to bloodstream. While an extensive list of bacterial systems that have global virulence regulating systems may be cataloged, our understanding of virulence regulation in the yeast *Candida albicans* is rudimentary.

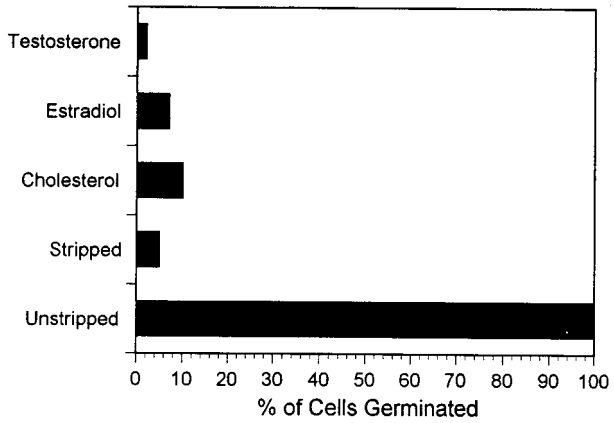

Figure 3. Effect of 1 μM steroid on yeast germination in stripped serum. A heavy suspension of yeast strain 49 was made in unstripped or stripped serum to which 1% ethanol had been added. The steroid compounds listed were diluted in ethanol and added to stripped serum. The final concentration of steroids was 1 μM and the final concentration of ethanol was 1%. Yeast cells were incubated at 37 °C for 180 minutes and the percent germination determined microscopically.

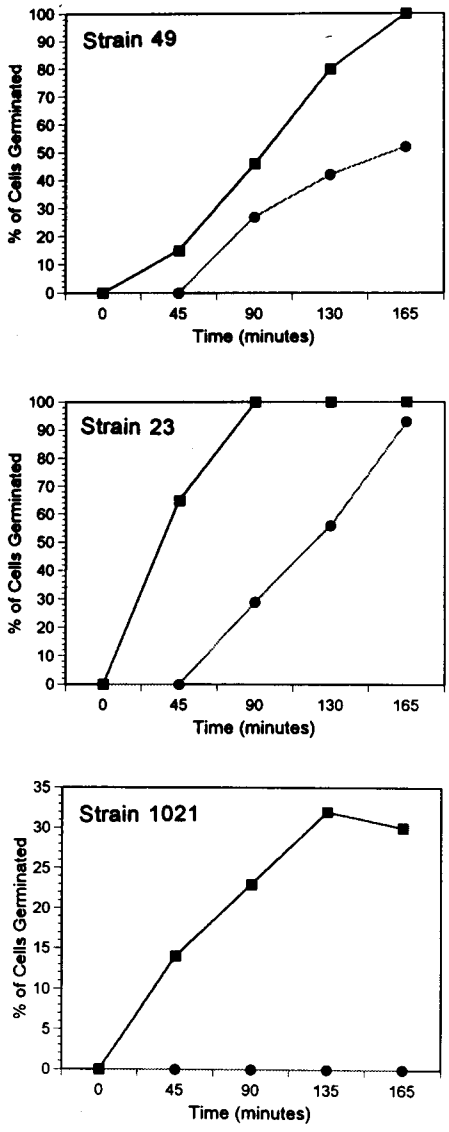

Figure 2. Time course of yeast germination in unstripped and stripped normal rabbit serum. Three yeast strains were added to aliquots of normal rabbit serum (solid squares) or serum that had been stripped with activated charcoal (solid circles). The percent of germinated cells was determined microscopically at regular intervals and plotted against time of incubation at 37 °C.

Although some uncertainty remains as to whether yeast to mycelial transition in *Candida albicans* is a true virulence factor [4, 8, 9], most investigators accept it as such since hyphal growth characterizes the organism in vivo. It is possible that mycelium formation may only indirectly promote virulence through cell surface hyrophobicity [10], the distribution of surface antigens [11], or by its interaction with endogenous or exogenous antifungal compounds [12]. Regardless of whether

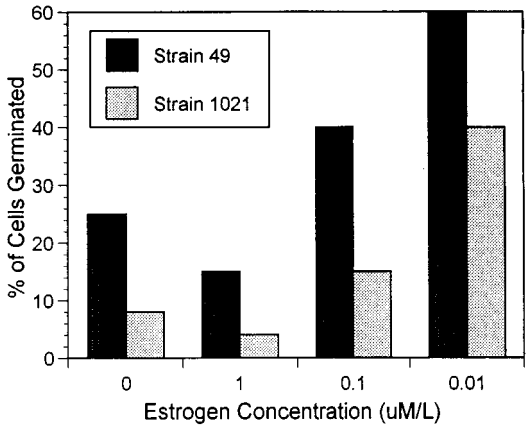

Figure 4. Titration of estrogen effect on yeast germination. Beta estradiol was diluted through serial 10-fold steps in stripped normal rabbit serum. Stripped serum with 1% ethanol was used as a control. A heavy suspension of each test organism was made in the estrogen dilutions and incubated at 37 °C for 180 minutes and the percent of cells germinated determined microscopically.

Table 1. Effect of combined steroid addition on yeast germination (strain 49).

Condition: Stripped normal rabbit serum	% of cells germinated at 90 minutes	% of cells germinated at 180 minutes
1% ethanol	2	19
Beta estradiol (2×10^9 M)	9	30
Cholesterol (2×10^9 M)	1	4
Beta estradiol (1×10^9 M) + cholesterol (1×10^9 M)	1	11

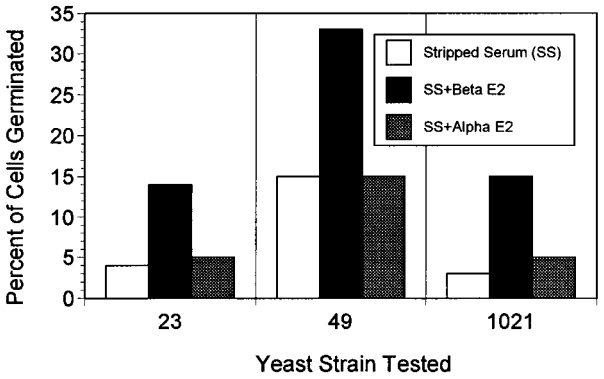

Figure 5. Comparison of germination regulation by alpha and beta isomers of estradiol. Stripped normal rabbit serum was supplemented with the alpha or beta forms of estradiol at a concentration of 1×10^9 M. Control stripped serum contained 1% ethanol. A heavy suspension of the test organisms was incubated at 37 °C for 180 minutes and the percent of cells germinated determined.

mycelial growth functions directly in virulence or is the indirect result of the organism adapting to growth within a susceptible host, myceliation may serve as a surrogate marker for the development of a pathogenic relationship with the host. Although several factors may elicit germination in vitro, the fact that there are agerminative mutants with reduced virulence [13] supports the concept of morphogenesis and virulence as coregulated phenomena.

A relatively young culture of the organism growing on artificial medium was almost entirely in the yeast form, but initiated morphogenic transformation in less than one hour in serum. We were able to remove a factor or factors from the serum by treatment with activated charcoal, although it was not within the scope of the present study to attempt a biochemical analysis of the compounds removed from the serum. Charcoal treatment of serum is not a specific procedure and may remove components other than estrogen, but its effectiveness in removing estrogen is clearly revealed by the loss of estrogenic activity in charcoal-treated serum as indicated by highly sensitive bioassays including the E-screen which uses cell cycling of human MCF-7 breast cancer cells [14] or the YES (yeast estrogen screen) assay in which the human estrogen receptor and a reporter consisting of two copies of estrogen response elements fused to the lacZ gene are cloned into *Saccharomyces*, making it responsive to natural and artificial estrogens [15, 16]. Our findings revealed that at least part of the germ tube stimulating activity was restored by the addition of beta estradiol to charcoal-stripped serum.

Somewhat unexpectedly, estradiol concentrations in the physiological range were more effective in promoting germination than were concentrations in the pharmacological range although this observation, coupled with the finding that the non-estrogenic alpha isomer of estradiol has little germination-promoting activity, is consistent with the possibility that *Candida* may use mammalian hormones to promote parasitism. Without identifying specific genes that are activated during mycelial transformation or elucidating their mode of regulation, it is impossible to describe the mechanism whereby micromolar concentrations of estradiol are less effective than nanomolar concentrations in producing germ tubes. However, in mammalian systems, steroid interactions with their cognate ligands do produce analogous results in terms of cell growth or enzyme activity as exemplified by studies done with breast cancer cell lines [17–19]. Such effects are believed to be due to downregulation of receptor production and hormone toxicity at hyperphysiological levels. *Candida albicans* has a cytoplasmic estrogen ligand, although this binding protein is not an analogue of the mammalian estrogen receptor [25]. Despite the difference in mammalian steroid receptors and the yeast estrogen-binding protein their dose-response relationships could be similar, even though different genes may be regulated in mammalian versus yeast systems.

Interestingly, the regulation of morphogenic transformation may be bidirectional, since Hazen and Cutler identified a naturally occurring substance from yeast which suppresses rather than induces mycelial growth [20]. The compound, which they named morphogenic autoregulatory substance (MARS) was not identified chemically, but it could be removed from their preparation by charcoal absorption. Zinc may serve as another factor that downregulates germ tube formation [21].

The idea that estrogen has a direct effect on yeast growth is not new. Estrogen is essential for successful colonization of rats with *Candida albicans* where it undergoes mycelial growth, although this effect is not necessarily a direct effect of estrogen [5]. Several fungal organisms in addition to *Candida* have steroid hormone-binding proteins [7, 22–24]. The estrogen-binding protein of

Candida, however, does not have homology with human estrogen receptor protein [25]. Recent studies in our laboratory quantified a tropic effect of 1 μM estradiol on yeast colony size, although this treatment failed to stimulate all strains and was actually inhibitory toward some [6]. In view of the current data, a greater degree of stimulation may have occurred if a lower estrogen concentration had been used in these experiments. Finally, estrogen has been identified as initiating cell cycle activity in yeast arrested in the G_0-phase [26].

While tropic effects of estrogen have been observed in various experimental situations, less is known about the ability of estrogen to promote morphogenic transformation. Kinsman and coworkers indicated that estriol, pregnanediol and pregnanetriol increased germination of *Candida* [27]. However, these investigators used serum diluted 1:10 as the medium to which test compounds were added. Whenever natural compounds are used in growth media there is always the possibility of steroid hormone being present and diluting serum tenfold will not obviate this problem. Even when plant-based growth media are used, the possible presence of phytoestrogens cannot be controlled. Our approach employed charcoal stripping to produce a hormone-free control serum. The data presented clearly indicate that estradiol promotes germination and that only the beta isomer is effective in this context.

The method of using stripped serum should prove useful in extending studies to other steroid compounds and to investigate whether the estrogen effect may be antagonized by other steroids. The results obtained by combining estradiol and cholesterol suggest that this may be the case and imply that virulence of *Candida* associated with hyphal forms may be upregulated or downregulated depending on the hormonal milieu on the host.

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