THE EFFECT OF THE MAMMALIAN HORMONE ESTROGEN ON THE YEAST *CANDIDA ALBICANS*

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LIST OF ABBREVIATIONS

AIDS		acquired immune deficiency syndrome
amino acids:	Y	tyrosine
	Q	glutamine
	L	leucine
	G	glycine
	R	arginine
bvgSA		*Bordetella* virulence genes S and A
C3dg		fragment of complement 3
CaURA3		*C. albicans* gene for orotidine-5'-monophosphate decarboxylase
CR3		complement receptor 3;(also named CD11b/CD18, Mo-1, Mac-1)
DNA		deoxyribonucleic acid
EBP		estrogen binding-protein
ECM		extracellular matrix
ERE		estrogen responsive element
Factor X		protein in the blood clotting cascade
FHA		filamentous hemagglutinin adhesin
gp63		glycoprotein with molecular weight of 63kDa
hCG		human chorionic gonadotropin
hER		human estrogen receptor
hLH		human leutininzing hormone

hsp	heat shock protein
iC3b	fragment of complement 3
ICAM-1	intercellular adhesion molecule-1
IgA	immunoglobulin A
LFA	leukocyte function-associated antigen
LPS	lipopolysaccharide
mAB	monoclonal antibody
MAPK	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RGD	peptide containing arginine, glycine, aspartic acid
RNA	ribonucleic acid
SAP	secretory acid proteinase
TAF	transactivating factor
toxR	toxin regulatory system
YCB	yeast carbon broth, contains carbon source, no nitrogen source
YEPE10	expression plasmid for hER
YNB	yeast nitrogen broth, contains nitrogen source, no carbohydrate source
YRpE2	reporter plasmid, ERE is the promotor for β-galactosidase expression

ABSTRACT

Title of Thesis: The Effect of the Mammalian Hormone Estrogen on the Yeast *Candida albicans*

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Changes in the balance between commensal microbes and mammalian hosts can cause disease if the scales tip in favor of the microbe. Although usually caused by a defective host immune response, overgrowth of or changes in the microbe may also be responsible for opportunistic infections. Clinical and laboratory investigations have indicated that mammalian hormones, including estrogen, can influence the production of symptomatic disease and the virulence attributes of *Candida albicans.*

This study demonstrated enhanced growth of some strains of *C. albicans* when 17β-estradiol was added to the growth medium. Measurement of extracellular protein under the influence of estradiol did not parallel the growth study results when corrected for the number of viable cells. Investigation of the effect of steroidal hormones on germ tube production, a putative virulence factor, showed no significant stimulation by any hormone. However, estradiol did induce four of the six strains to germinate more than their controls without hormone. The strains were variable in their innate tendency to germinate and in their response to steroidal hormones. Estrogen can protect mammalian cells from thermal stress suggesting

a study of estradiol's effect on the survival of *C. albicans* strains after exposure to 50°C for 15 minutes. Estrogen provided significant protection in four of the six strains tested. Each of these four strains showed significant suppression of acid proteinase secretion when grown 48 hours with estradiol when enzyme activity was corrected for cell number. Since *C. albicans* has a cytosolic estrogen-binding protein, we looked for genetic evidence of a structural homologue of the mammalian estrogen receptor. Southern blot analyses were performed on the genome of a *C. albicans* strain that had shown growth stimulation from 17β-estradiol using a 1.9kb fragment of the human estrogen receptor (hER) as a probe. No hybridization was detected under low or high stringency conditions. PCR was performed using primers based on a highly conserved portion of the hER with yeast genomic DNA as a template. No product was detected. The variability in response to estrogen between *Candida albicans* strains may be due to metabolic differences, genetic differences or structural differences. Although the mechanism of estradiol growth stimulation is unclear, such stimulation could have an influence on pathogenicity and therefore deserves additional investigation.

INTRODUCTION

Candida albicans, normally a benign, commensal yeast of the human mucosa and gastrointestinal tract, accounts for more deaths than all fungi combined (Komishian et al, 1985; Wey et al, 1988; Edwards, 1991; Saral, 1991; Jarvis and Martone, 1992) and causes an increasing percentage of infections among the expanding number of immunocompromised (both from chemotherapy and AIDS) and surgical patients (Meunier et al, 1981; Odds, 1988a; Butler and Baker, 1988; Kiehn et al, 1980; Horn et al, 1985; Cooper et al, 1988). Surveillance of intensive care units identifies candidiasis as the fourth most common nosocomial disease in the United States (Jarvis and Martone, 1992). The greatest number of infections are superficial, affecting mucocutaneous tissues such as the vagina or mouth. Although not life-threatening, this type of infection has significant morbidity. Millions of women worldwide are afflicted with candidal vaginitis which often proves refractory to treatment (Monif, 1985). Denture stomatitis is the most prevalent of the different types of oral infection and may be found in as many as 60% of elderly denture wearers (Odds, 1988a). The other group of candidal infections are the more serious, systemic or deep-seated diseases. Systemic candidiasis is rarer but has the highest mortality of the candidal diseases. How and why does *Candida albicans* cause disease? Understanding the factors contributing to *Candidal* pathogenicity to the mucosal surfaces can lead to better treatment of the more serious systemic diseases.

VIRULENCE

Webster's Dictionary defines virulence as "the relative infectiousness of a microorganism causing disease or ability to overcome the natural defenses of the host." The synergistic and additive nature of the organism's virulence properties forms one side of the equation. The host's defensive response is the other major factor in the equation. Although we know the defective response by the immunocompromised patient pushes the balance toward disease by *C. albicans*, the microbial factors important in virulence are less clearly understood. This thesis looks at the characteristics of *Candida albicans* that contribute to disease, especially in the non-immunocompromised host.

Virulence is usually thought of in terms of disease and therefore measured in terms of morbidity and mortality. But one must consider that the degree of host injury does not necessarily correlate with evolutionary success for the microbe. Survival and multiplication would seem to be the priorities for the microbe and disease is the result of the complex interactions required to accomplish these goals within the environment of the host. A broad view of virulence determinants includes all those factors contributing to infection as well as to disease. The exception would be the "housekeeping" functions which are required for efficient multiplication on nonliving substrates. Coordinate regulation of a housekeeping function with a definite virulence determinant can help support the function's role as a virulence factor (such as the regulation by iron of siderophore biosynthesis and cytotoxin production in *Corynebacterium diphtheriae*).

Environmental Signals and Regulation of Virulence Genes

More than one virulence determinant is typically involved in pathogenesis, as supported by numerous bacterial studies showing that specific virulence determinants contribute to unique steps in the pathobiology of microbes. Most of these studies looking at outright pathogens have established that a common regulatory system often controls expression of dissimilar virulence factors. This work has attempted to look at the normally commensal, but potentially lethal fungus, *Candida albicans*, from this same perspective. Inducing the expression of virulence genes probably involves the sensing of unique molecules produced specifically by the host tissues or by environmental cues that signal the entry of the microbe into host tissues. Host specific inducers, such as acetosyringone phenolic compounds and monosaccharides, trigger the expression of virulence factors in the phytopathogen *Agrobacterium tumefaciens* (Ankenbauer and Nester, 1990; Binns and Thomashow, 1988). Transcription of the ctxAB operon of *Vibrio cholerae* is affected by a number of environmental factors, including pH and low osmolarity. With a commensal organism these same signals may be used to detect anatomical differences at the tissue or cellular level. The studies presented in this paper looked at steroidal compounds, specifically 17β-estradiol (1,3,5(10)-estriene-3,17 beta-diol), as such a cue that might signal entrance into a host or changes that favor multiplication of the fungus.

Virulence genes in bacteria and plant fungal pathogens may be, on the one hand, those necessary for survival or growth in or on the living host, those

necessary to cause disease symptoms or, on the other hand, those that determine host-range (Gabriel, 1986). Investigations into bacterial pathogenesis have found a variety of virulence genes that function to sense their environment and are coordinately regulated to respond (Mekalanos, 1992; Larsen, 1994). Environmental conditions such as iron concentration, temperature, osmolarity, pH, oxygen, CO_2 or calcium can trigger a global or central regulatory gene which then acts as a master switch affecting several genes, and causing essential phenotypic changes in bacteria (Buchmeier and Heffron, 1990; Finlay and Falkow, 1989; Maurelli et al, 1992; Weiss and Hewlett, 1986). Similar environmental conditions may trigger fungi to upregulate virulence factors. The induction of acid proteinase production in *Candida albicans* by acidity and protein in the environment is an example (Staib, 1965; Bannerjee et al, 1991; White and Agabian, 1995).

The global regulation by the *toxR* transcriptional activation system in *Vibrio cholerae* that is regulated by iron availability (Goldberg et al, 1990, 1991) and the *bvg*SA (*Bordetella* virulence genes) central activation system of *Bordetella pertussis* (Clark, 1990; Miller et al, 1989; Weis et al, 1986) are some of the better described systems in bacteria. Transcriptional regulation is also a likely candidate for a governing point in eukaryotes, as studies in *Saccharomyces cerevisiae* have demonstrated (Struhl, 1989) . Work in *Saccharomyces* and the *alcR* regulon in *Aspergillus nidulans* indicates that regulons do exist in fungi (Davis and Hynes, 1991), despite the fact that none have been characterized in primary fungal pathogens yet. Finding a regulon controlling several virulence factors in fungal

pathogens is especially important since fungal pathogenicity is probably due to the sum total of several fungal factors and their balance against host factors.

Fungal Virulence

The details of pathogenesis have been studied more in bacteria than fungi. Unique entry or adherence factors, invasive properties or toxins have been recognized as harmful to the host and their corresponding genes have been referred to as "virulence genes" (Perfect, 1996). Since a fungal infection is due to an accidental encounter or a change in the parasite/host equation, factors such as the ability to adapt, survive and grow within the host are essential to the parasite's ability to produce disease (Steele, 1991). Genes controlling stress factors, such as heat shock proteins, may function as virulence genes in fungi (Matthews, 1994; Keath et al, 1989; Lathigra et al, 1991; Werner-Washburne et al, 1989) and may be just as important as those associated with more obvious virulence attributes such as proteinase production or capsule development. Certain housekeeping genes whose functions are required only for efficient multiplication on nonliving substrates are generally excluded from this virulence gene category. But even conserved genes with specific metabolic functions that are required only in vivo may be considered as contributing to virulence (Kirsch and Whitney, 1991; Shepherd, 1985). Thus, when considering fungi, it may be important to broaden the concept of virulence to include the unique ability of certain fungi to adapt, survive and grow within the host.

This ability is most likely due to the unique combination of traits found in a

particular species or even strain, not to single traits (Schreiber et al, 1985; Edison and Manning-Zweerink, 1988; Ray and Payne, 1990; Ray et al,1991). Cutler's virulence-set hypothesis states that "virulence traits belong to a set of genes within a given isolate expressing a finite number or subset of traits to make up the composite virulence phenotype of that particular strain" and that "a critical number of genes must act in concert to cause disease" (Cutler, 1991). In addition to this complex concept of pathogenicity, a set of genes may have the potential to change in importance, depending on the immune status of the host, the inoculum introduced, and the site of infection. This set-theory attempts to account for the genomic variability and the varied virulence properties among fungal strains and, if correct, makes the identification of the complete set of virulence genes even more complex.

The sensitivity of virulence genes to the environment makes selection of animal models especially critical and an understanding of the pathology of infection imperative. Certain virulence genes may be necessary to the establishment of infection following intravenous inoculation but may have little or no impact on mucocutaneous infections. Factors in the host-pathogen interaction that are important for the pathogenesis of disseminated candidiasis include access of the fungus to the intravascular space, penetration of the fungus to the interstitial space and protection of the fungus from the microbicidal action of leukocytes (Klotz, 1992a). In contrast, the attributes necessary for vaginitis may include adherence to epithelial cells, mycelium production, acid proteinase secretion (which degrades

IgA at the mucosal surface) and suppression of other normal flora permitting fungal proliferation beyond a certain critical mass (Van der Waaij, 1989).

The most virulent of the *Candida* species, *Candida albicans*, is an asexual, diploid fungus existing in at least two distinct morphological forms, long hyphal forms and spherical yeasts (blastospores). Both forms are present in the commensal and disease states. This ability to change morphology is considered to play a role in *C. albicans'* evasion of destruction by the host immune system. Phenotypic switching, evidenced by variant colony morphologies on agar plates, has been suggested as another important adaptive change that signals differential virulence. Expression of surface structures which are regulated by environmental conditions and decrease phagocytosis of the fungus may also contribute to virulence (Lehrer and Cline, 1969). Production of extracellular hydrolytic enzymes such as proteinases and phospholipases have also been suggested as putative virulence factors which aid adherence and invasion of the epithelium. Toxins and a hemolysin which contribute to the infectious process are attributed to *C. albicans*. All of these attributes and their contribution to disease will be described in this paper.

Pathogenesis or Détente

A pathogenetic pattern proposed for *Candida* infection involves adherence to and multiplication on mucosal surfaces, morphogenesis, and the production of excessive quantities of short-chain carboxylic acids as by-products of sugar metabolism (Samaranayake and MacFarlane,1985; Tomsikova et al, 1986). The

resultant acidic environment could cause direct irritation of the mucosal surface leading to inflammatory responses. This acidic milieu would also activate the acid proteinases of *Candida* leading to the cleavage of secretory IgA. Intact IgA can prevent *Candida* adhesion to epithelial cells (Epstein et al, 1981). This acidic condition can activate phospholipases of *C. albicans* which are able to destroy the host cell membranes (Pugh and Cawson, 1975). This model also credits this metabolically-induced acidic milieu with supporting the growth of acidophilic bacteria like lactobacilli and with inhibiting commensals that prefer a neutral pH. An acidic environment is reported to enhance the adhesion of *Candida* to epithelial cells (Samaranayake and MacFarlane, 1982).

This model may be true for the oral cavity, but the pH of the estrogenized vagina is normally acidic (pH 3.5-4.5). Even the pH of the estrogenized but uncolonized vagina (such as a newborn girl) is acidic (Weinstein et al, 1936). Although there is a commonly held belief that facultative lactobacilli also contribute to the normal acidic environment, research has not been able to demonstrate an absolute correlation between vaginal pH and the presence or absence of lactobacilli (Lewis and Weinstein, 1936; Weinstein and Howard,1939,1937). Facultatively anaerobic lactobacilli found in healthy, asymptomatic women, have demonstrated some protective antibiosis, perhaps through the production of hydrogen peroxide (Mårdh and Soltesz, 1983; Nagy et al, 1991). Other commensal vaginal microbes are tolerant of a broad range in pH (Galask, 1988; Larsen et al, 1976; Mårdh, 1991). If a strain of *Candida* colonizing the vagina is capable of acid proteinase

induction, the normal pH of 4 is ideal and no further acidification is required for the induction of acid proteinase.

Given the variety of phenotypic characteristics and the high-frequency switching phenomenon found among commensal and infective strains of *Candida*, it is possible that one strain can colonize under one guise and infect under another (Soll et al, 1987; Soll, 1988; Soll et al, 1988a,b; Soll et al, 1989). Switching affects such putative virulence factors as adhesion to epithelial cells, acid proteinase secretion, and morphogenesis, as well as aspects of cellular physiology such as permeability, sterol and lipid composition, cytoplasmic vacuoles, drug sensitivity, and sensitivity to neutrophils and in vitro oxidants (Soll, 1992; Ghannoum et al, 1990b; Lane and Garcia, 1991). In a study of *Candida* carriage at different anatomical sites of healthy women, 7 of 11 cases had genetically unrelated *Candida* strains in the oral cavity and vaginal canal. The other four cases had genetically similar but nonidentical strains from the same two sites (Soll et al, 1991). Similar results were obtained by other researchers demonstrating that isolates from different body locations were similar but not identical (Scherer and Stevens, 1988). It appears that the anatomically separated populations do not mix and have adapted to alternative environmental niches. A larger comparison between strains recently isolated from the same body site and after several laboratory subcultures might demonstrate if certain strains or groups of strains with similar DNA fingerprints and/or phenotypes tend to be oral-tropic or vaginotropic.

Since *Candida* is asexual, switching has been proposed as a mechanism for

differential gene transcription, and the resulting variability allows for rapid adaptation to changing culture conditions and host sites (Slutsky et al, 1987; Rikkerrink et al, 1988).

Microbe to Host Messages

There is also evidence that the normal flora affects the regulation within the cells of the host. Authors explain one interaction between a member of the normal mouse intestinal flora (*Bacteroides thetaiotaomicron*) and the intestinal epithelium (Bry et al, 1996). *B. thetaiotaomicron*'s normal use of L-fucose as a carbon source induces the messenger RNA of an essential transferase necessary for the fucosylation of glycoconjugates in the epithelial cells.

Although this is a model of the intestinal ecosystem, similar themes are likely to be present in the vaginal microbial ecosystem. Both tissues have continuously shedding epithelium (Gordon and Hermiston, 1994). However, the gut is highly differentiated with many different niches, secretory cells associated with the epithelial cells, and different maturation and renewal patterns between the various niches. The vaginal epithelium is much more homogeneous, with a more consistent, though cyclical, maturation rate. Vaginal secretions originate from glands in the cervix and outside the vagina, not from the vaginal tissue itself. Despite these differences, both tissues contain resident (autochthonous) microbial species and a variable set of transient (allochthonous) species that temporarily occupy niches (Savage, 1972, 1977). Disruption of this ecosystem with such interventions as antibiotics or immunosuppressive drugs has revealed the essential

role played by the microbiota in preventing infectious diseases (van der Waaij, 1989). Integration of the ecosystem into the host may be achieved through dynamic interactions that allow microbes to modify cellular differentiation programs, creating favorable niches. By regulating such pathways as fucosylation, organisms could affect the ability of other components of the normal flora to attach to epithelial cells (Hultgren et al, 1993) or obtain nutrients (Salyers and Guthrie, 1988). Evidence for the production of specific proteins in host cells after *C. albicans* adheres to buccal epithelial cells implies the same interaction with the host that has been demonstrated in the bacterial-intestinal epithelium model (Bailey et al, 1995). The vulnerability of the tissue to colonization by pathogens could also be affected.

The interplay between microorganisms of the normal vaginal flora, which may include *C.albicans* and gram-negative (various members of *Enterobacteriaceae*) and gram positive bacteria (ie. streptococci, staphylococci, lactobacillus), can be seen in the effect that lipopolysaccharide (LPS), from gram-negative bacteria, has on the phagocytosis of *C. albicans*. LPS has been shown to markedly augment phagocytosis of *Candida albicans* cells by increasing the number of yeasts ingested per neutrophil as well as the number of neutrophils capable of ingesting fungal cells. LPS also stimulated the release of several cytokines and lactoferrin from the neutrophils (Palma et al, 1992). This counterbalance between organisms is seen in clinical studies which have shown that gram-negative bacteria predominate in the absence of *C. albicans* (Auger and Joly, 1980).

Given these findings, it may be to *C. albicans'* advantage to maintain the population of gram-negative bacteria at a minimal level as is found in normal, asymptomatic, nonpregnant women (Tashjian et al, 1976; Sauter and Brown, 1980; Levison et al, 1979). The secretion of immunomodulating toxins like gliotoxin that inhibit chemotaxis of neutrophils is also obviously to *C. albicans'* benefit (Shah et al, 1992).

Candida albicans may create and take advantage of micro-niches to secrete the putative virulence factor acid proteinase, which requires a pH of less than 5.0 and the presence of protein.

Identifiying molecular interactions that confer stability between indigenous microbes and host cells can give us insights into how some commensal microorganisms start to cause disease and what goes awry when pathogens are introduced and they gain control (Ekenna and Sheretz, 1987; Hilton, 1995). One possible factor is cell density of the potential pathogen and the resulting feedback that triggers the production of virulence factors. This has been demonstrated in the global regulation of virulence factors by secreted lactones produced by *Pseudomonas aeruginosa* (Passador et al, 1993) . In the *B. thetaiotaomicron*-intestinal epithelium system, a critical density may be necessary to trigger epithelial fucosylation. This may reflect either a microbial signal that must reach a critical threshold, perhaps a breakdown product of L-fucose that feeds back to release another messenger, or a density-dependent change in the bacteria's metabolic properties, such as secretion of a hydrolytic enzyme involved in the utilization of L-

fucose.

Virulence factors are seldom produced by the normal microflora. One possible scenario has host signals, like glucose concentration and hormone levels, increasing *C. albicans* growth to a certain level, where a cell density-sensing mechanism starts to upregulate certain virulence factors.

Perhaps it would also be fruitful to approach the disease produced by "normal" flora by considering the microbes as a part of the host and looking at what triggers the inflammatory response in normal tissue. Studies of the dialogue between host and microbial ecosystem have looked at the mRNA and proteins produced when this dialogue changes and at the role of cytokines in the candidal inflammation.

Infections caused by different microbial pathogens elicit distinct patterns of inflammatory responses. This is due in part to variations in the profile of leukocyte adhesion molecules used and the cytokines released. In vitro, *Candida albicans* stimulates endothelial cells to synthesize and release prostaglandins (Filler et al, 1991; Filler et al, 1994), interleukin-8 (IL-8) and interleukin-6 (IL-6) (Filler et al, 1996). The increased surface expression of E-selectin and ICAM-1 on these endothelial cells, as well as the secreted IL-8, probably mediates the recruitment of neutrophils to infected areas (Xu et al, 1994; Bevilacqua et al, 1989; Smith et al, 1991). IL-8 has been shown to enhance the ability of neutrophils to kill *C. albicans* in vitro (Djeu et al, 1990). Although E-selectin and ICAM-1 are not known to be expressed by *C. albicans*, other analogs to leukocyte integrin receptors like CR3

are expressed by *C. albicans* and may interfere with this recruitment of neutrophils. However, it remains to be shown if this expression pattern of adhesin molecules and IL-8 is produced in vivo.

IL-6 is known to be elevated in mice with hematogenously disseminated candidiasis (Steinshamn and Waage, 1992). IL-6 stimulates B-cell activity (Hilbert et al, 1989), induces acute-phase reactants (Castell et al, 1989), and enhances the effects of TNF-α and platelet-activating factor on neutrophil function (Biffl et al, 1994; Mullen et al, 1995).

When messenger RNA for E-selectin, ICAM-1, IL-8, and IL-6 were measured and compared to their expressed and secreted proteins, it appeared that *C. albicans* was a stronger stimulus for mRNA expression than it was for protein secretion. It may be that proteases secreted by *C. albicans* degrade the secreted cytokines and reduce their accumulation (Filler et al, 1996). One recent outstanding discovery was that only conditions associated with candidal injury of endothelial cells stimulated the accumulation of endothelial cell mRNA for cytokines (Filler et al, 1996).

One host cytokine, IL-2, has been shown to bind to the mannoprotein of the cell wall in *C. albicans* blastospores (Treseler et al, 1992). Such binding by IL-2 has the potential to affect the cell mediated immune response of the host by triggering fungal killing by neutrophils. Alternatively, IL-2 bound and then released from the fungal surface could invoke a nonspecific immune response leading to inflammation which damages host tissue without effectively destroying the fungus.

The fungi could also remove soluble IL-2 before it reaches the intended target of the cell-mediated immune system.

Host to Microbe Messages

All of the parts of the nonspecific and specific immune response of the host are designed to eliminate invading organisms and contribute to the balance between host and commensal microbe. This is especially true with commensal organisms that cause serious disease when the host side of the balance is impaired. Host mechanisms that prevent adherence, limit nutrients such as iron and calcium, and target foreign cells for phagocytosis are well known. These protective host responses may trigger the production of virulence factors, as seen in the regulation of diphtheria toxin production by host iron levels. The adherence of the microorganism to the host can provide cues for bacterial pathogens to upregulate virulence factors. Recent work has proposed models of contact-dependent virulence gene regulation for uropathogenic *Escherichia coli* and *Yersinia pseudotuberculosis* (Zhang and Normark, 1996; Pettersson et al, 1996). For *C. albicans*, an acidic, protein rich niche in host tissue may trigger the production of the putative virulence factor acid proteinase.

Mammalian hormones may directly and indirectly affect the growth of *C. albicans*. Besides the clinical data indicating a candidiasis-hormone connection, work has also been done in animal models. A predisposition to vaginal candida infection has been noted in mice (Lehrer et al, 1983; Taschdjian et al, 1960) and in rats (Larsen et al, 1977) either during estrous or after ovariectomy and estrogen

administration (pseudoestrous). One explanation put forward is the greater adherence of blastospores to vaginal epithelial cells from animals in estrous or pseudoestrous (Lehrer et al, 1983; Segal et al, 1984). One other study in rats found that germ tubes were longer under estrous or pseudoestrous conditions than other hormonal states (Kinsman and Collard, 1986). This was also true when blastospores cultured in vitro were germinated in vaginal washings from rats dosed with various hormones (Kinsman and Collard, 1986).

Many cellular changes in the host, such as increased glycogen content and cornification of the epithelium, are induced in the vaginal environment by hormones. The degeneration of epithelial cells may release nutrients into the environment during the estrous period and this would contribute to increased growth of microbes. Indeed, higher bacterial counts in the vagina do occur during estrous in the rat (Larsen et al, 1977). In vitro investigations have shown that estrogen-treated HeLa cells had enhanced attachment of a variety of bacteria and that this process was energy dependent and involved protein synthesis (Sugarman and Epps, 1982). Genetic studies of pathogenic and commensal strains of *C. albicans* have indicated genetic and phenotypic differences (Scherer and Magee, 1990). DNA fingerprinting of *C. albicans* isolated from healthy and symptomatic patients has indicated that certain genetically similar groups of strains predominate the patients with symptomatic candidiasis (Schmid et al, 1995). Further testing demonstrated that these strains had significantly increased resistance to high sodium chloride concentrations (osmotic shock) and increased adherence to saliva-

coated surface. Adherence to host tissues is the first step of forming a microbe-host interaction, whether commensal or pathogenic.

VIRULENCE FACTORS OF *CANDIDA ALBICANS*

Adherence

Adhesion of *Candida albicans* to surfaces is an essentially irreversible attachment (Kennedy, 1987). The extent and strength of the adhesion depends on the initial surface properties of both the organism and substratum involved and can be influenced by several long-range, short-range, and hydrodynamic forces (Rutter, 1984). Short-range attractive forces, such as ionic, hydrophobic or dipolar interactions, contribute to nonspecific adhesion. Specific adhesion requires a "lock-and-key" type of mechanism involving interactions between complementary molecular configurations on the substratum and cell surfaces (Rutter, 1984). Any microbial surface macromolecule that mediates specific adhesion to a substratum surface receptor is referred to as an adhesin (Jones and Isaacson, 1983). Receptors are elements on the substratum surfaces which bind specifically to the "active sites" of microbial adhesins (Jones and Isaacson, 1983). *Candida albicans* cells adhere to a wide variety of host surfaces and tissues, including epithelial and endothelial cells, fibrin-platelet matrices, neutrophils, denture acrylics and plastics, extracellular matrix and laminin (Rotrosen et al, 1986; Douglas, 1985; Calderone, et al, 1991).

Arguments for adhesion as a virulence factor are mostly correlative (Scheld et al, 1981; Scheld, 1985, Ray et al, 1984). *Candida* species with a greater

pathogenicity potential also adhere better to buccal epithelial and vaginal epithelial cells than species which are rarely pathogenic (Kearns et al, 1983). *Candida albicans* and *Candida tropicalis* adhere to host cells in vitro better than the relatively nonpathogenic species such as *Candida krusei* and *Candida guillermondii* (King, 1980; Macura et al, 1983; Macura, 1985; Ray et al, 1984; Tomsikova et al, 1986). *Candida* strains having reduced adherence properties, either naturally or because of the conditions of growth or because of induced mutation, are less virulent in a rabbit endocarditis model (Calderone et al,, 1985; Douglas, 1987; Lehrer et al, 1986). *Candida albicans* strains isolated from women symptomatic for vaginitis are more adherent to vaginal epithelial cells than strains isolated from asymptomatic carriers (Segal et al, 1984). In the white/opaque strains of *C. albicans*, white colony cells are more adhesive to buccal epithelial cells (Kennedy et al, 1988; Kennedy et al, 1989).

Although we don't know if adherence is the only factor contributing to these strains' pathogenicity, investigators have decreased susceptibility to disease in mice by blocking adherence in vivo with candidal extracts. A chitin soluble extract prepared in a cream and applied vaginally to mice in estrus reduced the establishment of *Candida* vaginitis (Lehrer et al, 1983; Segal et al, 1984; Taschdijian et al, 1960), and a chitin extract administered intravenously (iv) prolonged survival in mice subsequently challenged with *Candida albicans* iv (Suzuki, 1984). When rabbits were administered *C. albicans* iv and then given a synthetic arginine-glycine-aspartic acid (RGD) containing-peptide iv, there was a

reduction in recoverable organisms from tissue (Klotz et al, 1992b; Sawyer et al, 1992). Since quantitative blood cultures were not reported, the possibility exists that the RGD peptide entrapped the organisms within the vascular bed by preventing their migration into the tissues, evidenced by similar fatality rates between peptide-treated and untreated animals. Yeast attachment to vascular endothelium was also greatly reduced in vitro when pretreated with RGD peptides (Klotz, 1990).

The prevention or interruption of candidal adherence to mucosal or vascular tissue in vivo could affect the development of disseminated and superficial candidiasis (Burke and Gracey, 1980). Recently published studies attribute an even more direct role of adherence in bacterial virulence. These studies show that adherence of the bacteria *Yersinia pestis* and uropathogenic *Escherichia coli* to host cells triggers the expression and secretion of well-known virulence factors (Pettersson et al, 1996; Zhang, 1996). Preliminary studies with *C. albicans* indicates that adherence to human buccal epithelial cells (HBEC), in vitro, triggers phosphorylation of proteins and specific protein synthesis (Bailey, 1995). If the implications of the bacterial research are also true in *C. albicans*, adherence not only keeps the organism in the host so that it can multiply but activates other virulence factors which aid in the infectious process.

Considering that *Candida albicans* colonizes host mucosal surfaces that are constantly bathed with fluids and are continuously shedding epithelial cells, attachment to the host surface becomes an important issue in *Candida* infections

(Edwards and Mayer, 1990). However, the association of *C. albicans* with any host tissue is a multifactorial process involving all or several virulence mechanisms and the individual contribution of any one factor may be expected to vary with environmental conditions, host conditions, and strain of *Candida* (Garcia-Tamayo et al, 1982).

Factors in the host that influence candidal adherence in vivo include the type of animal model used in a study, hormonal status, cell type, immunologic status, antimicrobic therapy, underlying debilitating diseases, environmental factors (e.g. pH, bile and fatty acids), diet, and body site (Freter and Jones, 1983; Cole et al, 1990; Segal et al, 1984; Kalo and Segal, 1988; Kinsman and Collard, 1986; Kinsman et al, 1988; Sandin, 1982, 1987; Segal et al, 1984; Epstein et al, 1982; Lehrer et al, 1983; Scheld et al, 1983; Vudhichamnog et al, 1982; Kennedy and Volz, 1985a, 1985b; Kennedy, 1987; Odds, 1988a; Persi et al, 1985; Samaranayake and MacFarlane, 1982, 1985; King et al, 1980). The indigenous microflora of the host also affects *Candida*'s adhesion by preemptive colonization of adhesion sites and modification of the substrate, through the production of inhibitors, and alteration of the physiochemical nature of the host's microenvironment (Kennedy and Volz, 1985b; Liljemark and Gibbons, 1973). The surface of the host epithelial cells changes with various physiological conditions, such as HIV infections (Sweet, 1995).

Adherence is not just a single event, but a series of closely related phenomena, including the approach of the fungus to the adherence target, loose adherence of

the fungus to the tissue, and tight adherence over time (Klotz, 1992a; Rotrosen et al, 1986). Yeast factors that contribute to adhesion include species and strain, viability, germ tube formation, enzyme production (e.g.. proteinase, phospholipase), concentration and type of adhesins, phenotypic switching and cell-type, coadhesion, and surface properties (e.g.. hydrophobicity) (Critchley and Douglas, 1985; King et al, 1980; Klotz et al, 1983; McCourtie and Douglas, 1984; Miyake, et al, 1986; Rotrosen et al, 1985; Scheld et al, 1983; Kimura and Pearsall, 1978; Lee and King, 1983; Samaranayake and MacFarlane, 1980; Sobel et al, 1981; Kimura and Pearsall, 1978, 1980; Sandin and Kennedy, 1987; Sandin et al, 1982; Anderson et al, 1985; Rotrosen et al, 1985; Samaranayake et al, 1981; Samaranayake and MacFarlane, 1982a, Samaranayake and MacFarlane, 1982b; Sobel et al, 1981; Barret-Bee, 1985; Ghannoum and Elteen, 1986; McCourtie and Douglas, 1985a,b; Kennedy et al, 1988, 1989; King et al, 1980; Sandin et al, 1987; Hazen et al, 1986; Klotz et al, 1985; Slutsky et al, 1987). The disruption of adherence of *Candida* to tissue, whether mucosal or vascular, in vivo could potentially abate candidiasis.

Although pathogenesis of any fungal infection involves a complex series of separate, interdependent interactions which cannot be duplicated in vitro, some useful in vitro models have been developed to identify, differentiate and study molecular adhesive mechanisms. The data generated must be interpreted with caution when applying them to in vivo situations however. At some point the in vitro mechanism must be demonstrated in vivo.

Extensive investigation in this area has evolved several systems for categorizing adhesin-host interaction (Cutler, 1991; Hostetter, 1994; Douglas, 1995). One category found in all systems is the protein-protein interaction in which a protein such as the integrin-like receptor for iC3b on the surface of *C. albicans* binds to RGD-containing host proteins, like iC3b (Gilmore et al, 1988; Bendel and Hostetter, 1993; Hostetter, 1994) and subendothelial matrix components such as fibronectin (Penn and Klotz, 1994; Klotz, 1994; Klotz et al, 1994; Klotz, 1990; Klotz and Smith, 1991; Skerl et al, 1984) or laminin (Bouchara et al, 1990; Klotz, 1990). A second class of receptor-ligand interactions is the lectin-type interactions between a protein of *Candida* and a carbohydrate in the host glycoproteins (Critchley and Douglas, 1987) or glycolipids (Tosh, 1992; Cameron and Douglas, 1996). A third proposed class of interaction remains to be thoroughly defined, but is typified by the factor 6 epitope of *C. albicans* which recognizes an unidentified host-cell membrane receptor-ligand (Miyakawa, 1992). A fourth interaction class described by Douglas (1995) is the interaction of fimbrial adhesins (Yu, 1994) whereas Cutler proposed a class to include hydrophobic interactions (Cutler, 1991; Hazen and Hazen, 1988; Hazen, 1989; Hazen et al, 1991). This diversity of adhesive interactions between organism and host adds to the complexity of defining the conditions which influence adherence.

It is reported known that a diet high in carbohydrates can predispose patients to oral candidiasis (Odds, 1988a). Dietary carbohydrate supplementation also affects colonization and invasion by *Candida albicans* in a neutropenic mouse

model, implying an effect on endothelial cell adherence (Vargas et al, 1993). These in vivo effects lead to in vitro investigations.

The composition of the growth media was found to greatly influence the various types of adherence interactions exhibited by *Candida albicans*. Investigators looking at the in vitro effect of various sugars in high concentrations found that high levels of galactose, sucrose, glucose and maltose increase adherence to acrylics when compared to cells grown in a medium containing a lower concentration of glucose (Samaranayake and MacFarlane, 1981, 1982a, 1982b; McCourtie and Douglas, 1981). The type of sugar also affects in vitro adherence and virulence of pathogenic strains (McCourtie and Douglas, 1984). The strains with a higher tendency to cause infection also demonstrate an enhancement of in vitro adherence when grown with galactose (Critchley and Douglas, 1985). Additional growth factors affecting in vitro studies of adhesion include viscosity of the medium , temperature, and phase of growth (log, stationary, etc) of *Candida* (Lee and King, 1983; Sandin et al, 1987; Segal et al, 1982; Segal and Savage, 1986; Anderson, 1985; King et al, 1980).

Environmental factors affecting adhesion assays include the assay medium, the assay temperature,, incubation time, yeast to epithelial cell ratio, and pH (Lee and King, 1983; McCourtie and Douglas, 1981; King et al, 1980; Kimura and Pearsall, 1978, 1980; Samaranayake et al, 1980; King et al, 1980; Persi et al, 1985; Samaranayake et al, 1982a; Sobel et al, 1981). More recent studies have shown that *C. albicans* cells grown in hemoglobin-containing defined media demonstrated

an increased adhesion to immobilized fibronectin as well as to endothelial cells (Yan et al, 1996). Host antibodies can block in vitro adhesion of *C. albicans* to human oral mucosa (Epstein et al, 1982; Lehrer et al, 1983; Vudhichamnog et al, 1982) Monoclonal antibodies have been used in adherence-blocking experiments to identify specific adhesins on the surface of *C. albicans* (Klotz, 1990; Hostetter, 1991). The results of the latter studies must be carefully evaluated, however. For instance, if two or more specific inhibitors block adhesion, it may indicate there is more than one adhesin or that different sites on the same multifactorial adhesin are present (Kennedy, 1987). Extrapolation of in vitro results to in vivo situations is perilous. In vivo epithelial cell surfaces are bathed by various secretions which may cause glycoproteins to bind to receptors of the epithelial glycocalyx. Thus, microbial cells may adhere in vivo not to receptors on the cell surface proper, but to ligands of tissue secretions coating the tissue surface or factors adsorbed to the surface (Freter and Jones, 1983; Gibbons et al, 1985).

Recent studies have demonstrated the upregulation of expression of the integrin-like receptor of *Candida albicans* that mimics a portion of the human complement receptor for iC3b (CR3, Mac-1). When the organism was grown in high levels of glucose, expression of the iC3b receptor increased and yeast cells showed an increased resistance to phagocytosis (Gilmore et al, 1988; Hostetter et al, 1990; Bagdade et al, 1972). The mammalian receptor, CR3 (CD11b/CD18), is found on human neutrophils, monocytes, large granular lymphocytes and follicular dendritic cells (Ross and Rabellino, 1979, Ross and Lambris, 1982). It is the major

phagocytic receptor for virulent pneumococci (Gordon et al, 1986), and is thought to have a central role in host defense against various microorganisms by triggering superoxide production and degranulation (Gordon et al, 1986). The integrin analog on *C. albicans* is thought to participate in yeast adhesion since substantial inhibition of adhesion to endothelial monolayers was noted after preincubation of yeast cells with iC3b, the RGD-containing ligand specific for the CD11b/CD18 (Gustafson et al, , 1991).

iC3b Receptor

The iC3b receptor expressed by *Candida albicans* shares antigenic and structural homology with the α-subunit of the leukocyte adhesion glycoprotein CD11b/CD18, also known as Mo1, Mac-1, or CR3 (complement receptor type 3), found on human neutrophils, large granular lymphocytes, follicular dendritic cells, monocytes, and macrophages (Ross and Rabellino 1979; Ross and Lambris 1982; Sanchez-Madrid et al, 1983; Anderson et al, 1986; Wright et al, 1983). An understanding of the composition and function of CR3 is necessary in order to hypothesize about the function of its homologue, the iC3b receptor, on the surface of *C. albicans*.

Neutrophil extravasation into tissues and phagocytosis of complement-coated particles are essential steps in host defense against infections. Complement receptor type 3 (CR3, CD11b/CD18, Mo-1, Mac-1) is a major cell surface glycoprotein necessary for circulating phagocytes to migrate into inflamed organs and to phagocytose opsonized particles (Kishimoto et al, 1989). Eukaryotic ligands

for CR3 are known to include coagulation Factor X, iC3b, and intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, and fibrinogen (Lo et al, 1991; Marks et al, 1989; Altieri and Edgington, 1988a; Altieri and Edgington, 1988b; Altieri et al, 1990; Diamond et al, 1990; Wright et al, 1983). It had been suggested that this combination of binding specificities provides a point of convergence of the pathways for leukocyte adhesion and coagulation which operate during inflammation (Altieri et al, 1991; Li et al, 1995). CR3, a member of the β2 integrins, is a heterodimer consisting of two noncovalently associated subunits, CD11b and CD18, with apparent molecular masses of approximately 160kDa and 94kDa, respectively (Hynes, 1987; Hynes, 1988). Other neutrophilic adhesins use the CD18 subunit with CD11a (LFA-1) or CD11c (p150,95) (Sanchez-Madrid et al, 1983). In general, all of these adhesion/receptors promote interaction of leukocytes with each other (Arnaout et al, 1985; Schwartz et al, 1985), with endothelial cells during transmigration (Diener et al, 1985; Arnaout et al, 1988; Luscinskas et al, 1989) and with specific opsonins (Arnaout, 1983, Klebanoff et al, 1985). CR3 is also the major phagocytic receptor for virulent pneumococci (Gordon et al, 1986) and the soluble heterodimer CD11b/CD18 can inhibit binding of neutrophils to activated endothelium (Dana et al, 1991). The importance of the β2 integrins is underscored in patients with inherited deficiencies of these integrins (Leu-CAM deficiency) which compromises the phagocytic and migratory capacities of circulating granulocytes and monocytes, leading to life-threatening bacterial infections (Arnaout, 1990; Lo et al, 1991). Monoclonal antibodies (mAbs) to the

CD11b or CD18 subunits of CR3 reproduce these defects in experimental animals, underscoring the major role of CR3 in these events (Arnaout, 1990; Elemer and Edgington, 1994).

Major opsonins of the humoral immune system are the products derived from proteolytic activation of complement component C3, the product of a 42kb gene on chromosome 19 (Hostetter, 1993). Cleavage of the native C3 by either the classical or alternative pathway C3 convertases releases C3a, a small peptide from the amino terminus of the C3 α-chain and produces C3b with an exposed highly reactive thioester bond (activation). The process of opsonization uses this thioester group for transacylation reactions with either free hydroxyl or amino groups on the surface of the microorganism, covalently bonding the C3b to the surface (Hostetter et al, 1982). It is subsequently degraded by serum protease factor I in the presence of cofactors such as soluble serum factor H or blood cell-associated complement receptor type I (CR1) first to iC3b and then to C3dg. Each of these C3 proteolytic fragments, which remain covalently attached to the target, exhibits unique structures that mediate interactions with numerous regulatory molecules and cell surface receptors and cause a variety of biological effects, such as the interaction between CR3 and FcRIII.

Synergy between CR3 and FcRIII (receptors for the Fc portion of IgG) on neutrophils can initiate a respiratory burst, which is diminished if either is blocked by monoclonal antibodies or if the actin cytoskeleton is inactivated by cytochalsain B (Zhou et al, 1994). Studies examining the effect of staurosporine, an inhibitor of

both protein kinase C and tyrosine kinase, on PMA-activated neutrophils and their CR3 expression showed that although CR3 expression increased (probably by translocation of intracellular stores), phagocytosis via CR3 was inhibited. Since FcR-mediated phagocytosis was not affected, different pathways are implicated (Roubey et al, 1991).

CR3's interaction with several ligands, iC3b, the major opsonic fragment of C3 (Arnaout et al, 1983), fibrinogen, extracellular matrix components (ECM), and factor X (Altieri, Edgington, 1988, Wright et al, 1988; Taniguchi-Sidle and Isenman, 1994) depends upon the presence of divalent cations. Short peptides containing the Arg-Gly-Asp (RGD) triplet can inhibit the attachment of cells by various integrins to extracellular matrix (ECM) ligands (Pierschbacher and Ruoslahti, 1984; Ruoslahti and Pierschbacher, 1987). The RGD sequence is present in the α-chain of C3 and early work indicated that CR3 did bind a 21-amino acid RGD-containing peptide from C3. A segment of this peptide also inhibited rosetting between iC3b-coated erythrocytes (EC3bi) and CR3-bearing neutrophils (Wright et al, 1987; Wright et al, 1988). However, experiments with site-directed mutagenesis of the RGD sequence in C3 indicate that the iC3b-CR3 interaction is not mediated by the RGD triplet or its neighboring residues (Taniguchi-Sidle and Isenman, 1992). It may be that the peptides bind to a site different from the iC3b binding site and modulate affinity for iC3b or that the conformation of the peptides mimics that of the active site on iC3b. The small size of the peptides (10 amino acids) makes stearic hindrance unlikely. β2 integrins do not utilize small linear peptide sequences to

bind but recognize multiple discontinuous sites on their adhesive proteins (Taniguchi-Sidle, 1992; Staunton et al, 1990; Diamond et al, 1991). The β subunits of Mac-1 are also implicated in ligand binding, with residues Asp^{134} and Ser^{136} playing essential roles in iC3b recognition (Bajt et al, 1995).

After monoclonal antibody mapping experiments implicated the importance of the A (I) domain of the α-chain of CR3 in ligand recognition (Diamond et al, 1991; Diamond et al, 1993b; Diamond and Springer, 1993a), elegant work by Ueda et al (1994) has narrowed the iC3b binding site of CR3 to between amino acid residues 232 and 245 in the extracellular A (I) domain of CD11b (α chain) . This 200-amino acid domain is proximal to three metal binding sites (Arnaout et al, 1988) and also contains a metal binding site (Michishita et al, 1993). This magnesium ion-dependent "MIDAS" (metal ion-dependent adhesion site) binds a critical aspartate (D) residue and β-looped structure in ligands termed "RGD-mimetic" (Lee et al, 1995, Bergelson, 1994). This MIDAS motif also forms the RGD binding region of the β3 chain, which clarifies how anti-β3 integrin monoclonal antibodies like 7E3 inhibit Mac-1 (CR3) (Lee et al, 1995; Zhou et al, 1994). It also explains why such diverse ligands as fibrinogen (RLD; Zhou et al, 1994), iC3b (De...EE; Taniguchi-Sidle, 1994), and factor X (GYD..QED; Rozdzinski et al, 1995) bind to Mac-1 (CR3). Other proteins containing the A (I) domain include cartilage matrix, von Willebrand factor and α_1 and α_2 subunits of the β1 integrins.

Other work demonstrated peptide binding to the α-chain (CD11b) (partly composed of the A (I) domain) of CR3 and that the LPS binding to the β-chain

(CD18) of CR3 (Wright et al, 1989) is via a phosphosugar (Wright and Jong, 1986). Binding of CD11b/CD18 to glucan (Georgepapdakou and Tkacz, 1995) and to zymosan, both polysaccharides found in yeast, is inhibited by N-acetyl D-glucosamine (NADG) and is mediated by a site distinct from the iC3b-binding site and proximal to the cell membrane (Ross et al, 1985; Arnaout, 1990; Anderson et al, 1986). The monoclonal antibody (mAB) OKM1 binds to an epitope in this lectin-like domain of CR3 (Diamond et al, 1993b) and does not block iC3b binding. A possible scenario for these two binding sites is that the lectin-like site may crosslink Mac-1 (CR3) into clusters of receptors resulting in "activation": quantitative and qualitative changes such as release of integrin from intracellular stores and aggregation on the cell surface (Detmers et al, 1987), association with cytoskeleton (Rabb et al, 1993) and changes in conformation with the expression of specific neoepitopes (Forsyth, 1996). The RGD mimetics then adhere, resulting in intracellular signaling (Graham et al, 1994; Zhou et al, 1994; Forsyth, 1996). This receptor clustering and ligand occupancy can synergize during integrin adhesion (Miyamoto et al, 1995; Zhou et al, 1994). CR3 (Mac-1) is probably a broadly specific receptor for the polysaccharides on the surface of microorganisms which also have iC3b bound or some other RGD mimetic. It also binds virulence factors such as the RGD-containing filamentous hemagglutinin (FHA) glycoprotein of *Bordetella pertussis* (Relman et al, 1990). *Leishmania mexicana* expresses two distinct structures that bind Mac-1 (CR3) : a surface glycolipid (LPG) that can be blocked by OKM1 and not RGD and a second protein (gp63, with RYD) which binds

in an RGD-inhibitable manner (Talamas-Rohana et al, 1990; Russell et al, 1989). Extracellular matrix proteins containing RGD can inhibit adhesion of large granular lymphocytes (LGL) expressing Mac-1 to *Candida albicans* hyphae (Forsyth, 1996) but the tertiary structure is important as a circular molecule containing RGD had no effect on adhesion (Pierschbacher and Ruoslahti, 1987).

Serendipity played a role in the discovery of the iC3b receptor on *Candida albicans* when complement-coated sheep erythrocytes (EAC) rosetted contaminating yeast cells in a lymphoblastoid culture. Investigation revealed that only iC3b- and C3d-coated erythrocytes bound to the pseudohyphae. Among the five species tested, only *Candida albicans* and *Candida stellatoidea* showed adherence, paralleling the pathogenicity of the different species (Heidenreich and Dierich, 1985). Further evidence for the role of this iC3b-receptor in virulence accumulated when a spontaneous avirulent mutant (resistant to cerulenin) was found to have reduced in vitro adhesion to fibrin platelet clots and epithelial cells, as well as a reduced ability to cause endocarditis in the rabbit model (Calderone et al, 1985) and to cause vaginitis in a murine model (Lehrer et al, 1986). This mutant was also found to bind iC3b-coated erythrocytes to a lesser extent that its wild-type parent, (Ollert, 1990). Total cell extract could not inhibit the iC3b-mediated rosetting, indicating that a functionally inactive receptor or a greatly reduced production not just expression on the surface of *Candida* was responsible for the inability to bind iC3b (Ollert et al, 1990). Other in vitro studies with monoclonal antibodies to CR3 (the human receptor for iC3b) blocked adherence

of *Candida albicans* to iC3b and to human endothelial cells (Gustafson et al, , 1991). The serum of a patient with chronic mucocutaneous candidiasis bound this iC3b-receptor on an immunoblot, suggesting immunogenic properties and perhaps a role in virulence (Ollert et al, 1990). Increased expression has also resulted in decreased phagocytosis (Hostetter et al, 1990) and blockade of the integrin analog on blastospores with the IgM monoclonal antibody anti-Mo-1, significantly improved phagocytosis of yeast cells by normal human neutrophils (Gilmore et al, 1988). This receptor is not an artifact of in vitro conditions because expression has been seen in immunofluorescence studies in which multiple forms (yeast cells, germ tubes, and pseudohyphae) of *C. albicans* were recovered form the peritoneal cavity of mice and stained with OKM1 (Kanbe et al 1991). All of this indicates a role of this receptor for iC3b in the pathogenesis of *Candida albicans*.

Early characterization of the iC3b-receptor of Candida indicated it was expressed only on pseudohyphae in a temperature-dependent and cation-independent manner (Eigentler et al, 1989). Immunoprecipitation showed a single chain structure with a molecular weight of 145 kDa, similar to CD11b of CR3. Staining with only monoclonal antibodies to the α-chain of CR3 and not to the β-chain of CR3, as well as inhibition with RGD-containing peptides further indicated homology to the human CR3 (Eigentler et al, 1989). This homology has been further explored with other monoclonal antibodies that are specific for the α- or β-chain of CR3. Only antibodies against the α-chain (such as Mo1, M1/70, and OKM1) react on immunoblots of surface proteins or block the interaction of iC3b

and the receptor (Mayer et al, 1990; Alaei et al, 1993).

In contrast to CR3, iC3b binding to the *Candida* receptor protein is independent of cations (Eigentler et al, 1989). However, some researchers have found that calcium is necessary for long-term stabilization of the interaction on pseudohyphae (Spotl et al, 1994). The interaction of the extracellular matrix proteins type I collagen and fibronectin and *C. albicans* is dependent upon calcium (Klotz et al, 1993).

The first researchers to discover the iC3b-receptor on *C. albicans* also tested various carbohydrates for their ability to inhibit interaction between the receptor and the complement fragment. Only D-glucose and D-mannose inhibited, suggesting a lectin-like nature to the interaction between *Candida albicans* and iC3b-coated erythrocytes (Heidenreich and Dierich, 1985). Although iC3b contains the RGD tripeptide, purified fibronectin or fibronectin-RGD peptides failed to block candidal adhesion to epithelial cells. In contrast, 9-15 mer RGD peptides from iC3b all inhibited *C. albicans* adherence to epithelial cells (Bendel and Hostetter, 1993). *C. albicans* adheres to immobilized components of the extracellular matrix, including Type I & IV collagen, fibronectin, and laminin. Fibronectin inhibited candidal binding to all four substrates, however RGD peptides did not reproducibly inhibit binding to collagen or laminin (Klotz and Smith, 1991). All this suggests that the receptor-ligand interactions of *Candida albicans* differs between epithelial cells and extracellular matrix. The flanking sequences of the RGD peptide apparently give specificity to the interaction. Carbohydrates also affect the expression of

this iC3b receptor by *C. albicans*. Growth in 20 mM D-glucose, as opposed to 20mM L-glutamate, increased expression by 25.0% as measured by flow cytometric analysis with monoclonal antibodies recognizing the α-subunit of CD11b/CD18. This also correlated with a significant increase in adhesion of glucose-grown *C. albicans* to endothelial cells (Gustafson et al, 1991).

Just as increases in ambient temperature to 37°C result in increased expression of CR3 in the neutrophil, the expression of its analogue in *C. albicans* is also increased at 37°C (Gilmore et al, 1988; O'Shea, et al, 1985; Eigentler et al, 1989). The binding kinetics of iC3b and the integrin analogue of *C. albicans* are virtually identical to that established for its interaction with the CR3 on the neutrophil (Gilmore et al, 1988; Gordon et al, 1987). Thus this integrin analogue of *C. albicans* is considered a true receptor for iC3b (Gustafson et al, 1991).

Where the CR3 of the neutrophil consists of a β subunit of Mr 95kD common to all members of the leukocyte adhesion glycoprotein family, and a specific α subunit of Mr 165kD (Sanchez-Madrid et al, 1983), studies with monoclonal antibodies indicate that the analogue of *C. albicans* has only an α-subunit with Mr of 130-165 kD (Hostetter et al, 1990; Eigentler et al, 1989), with homology with the α-chain of CR3 (α_m). Although one study isolated several proteins (70, 66, 55, 42 kD) from disrupted pseudohyphal forms of *C. albicans* by affinity chromatography with a biologically modified C3 molecule that is conformationally similar to iC3b, and these proteins cross-react with OKM1 (a monoclonal antibody to α_m) on Western blots, the 42-kD protein appears to have no homology with the structure of the human α_m

(Eigentler et al, 1989). Sequencing of the amino terminus or internal peptides and isolation of the gene(s) will be required to show the derivation of the 42-kD protein and its relationship to candidal integrin analogs.

The gene for an integrin-like protein has just recently been isolated from a *C. albicans* genomic DNA library by screening with a cDNA probe from the transmembrane domain of human α_m (Gale et al, 1996). The predicted polypeptide (Int1p) of 188kD contains several motifs common to α_m and α_x (α-chain of CR4): a putative I domain, two EF-hand divalent cation-binding sites, a transmembrane domain, and a cytoplasmic tail with a single tyrosine residue. An internal RGD tripeptide is also present. Despite these similarities to α_m Int1p is considerably larger and the sequence of the I domain and divalent cation-binding motifs are different. Binding of anti-peptide antibodies raised to potential extracellular domains of Int1p confirms surface localization in *C. albicans* blastospores. Southern blotting showed that INT1 is unique to *C. albicans*. Expression of INT1 under control of a galactose-inducible promoter led to the production of germ tubes in haploid *Saccharomyces cerevisiae* and in the corresponding *ste12* mutant. This morphological change may be caused by a disruption of the cytoskeletal architecture or of the growth cycle by Int1p. Anti-α_m monoclonal antibody also bound to the surface of these *S. cerevisiae* tranformants. This certainly leads to speculation as to the function of Int1p in *Candida albicans* especially regarding pathogenesis, signal transduction and differentiation. Studies of mRNA expression under different growth conditions, pathogenicity of null-mutants, and signal

disruption will clarify these questions.

One of the growth conditions to be tested is the presence of steroid hormones. Regulating the number of receptors is one mechanism steroids use to affect cells. Hormones can control growth factor receptor expression in mammalian cells (De Bartoli et al, 1996). Estrogens can increase the number of thyrotropin-releasing hormone receptors (Gershengorn et al, 1979), tumor necrosis factor receptors are regulated by retinoic acid (Chambaut-Guerin et al, 1995) and dexamethasone (Chambaut-Guerin and Thomopoulos, 1991), and estrogens inhibit erbB-2 (a growth factor receptor) expression by transcriptional repression (Bortoli, 1996). 17β-estradiol has also been shown to inhibit cytokine-mediated endothelial cell adhesion molecule transcriptional activation for E-selectin, vascular cell adhesion molecule-1 and intercellular adhesion (Caulin-Glaser et al, 1996). This ability to change the surface molecules of mammalian cells may extend to the eukaryote *C. albicans*, which is commensal to mammalian cells and expresses an integrin analog, iC3b receptor, on its surface.

Why does *Candida albicans* have an analog of the human CR3? What selective advantage or purpose does it serve? *C. albicans* is not the only microorganism to co-opt an existing integrin-based system of receptors and ligands. *Histoplasma capsulatum* (Bullock and Wright, 1987), *Bordetella pertussis* (Relman et al, 1990), *Leishmania major* (Mosser and Edelson, 1985), *Escherichia coli* (Sharon et al, 1991) and *Mycobacterium tuberculosis* (Schlesinger et al, 1990) use the integrin system to evade mortal phagocytosis. One strategy used could be called masking

(Falkow, 1991), where adsorption of the natural ligand for the integrin expressed on the surface of the pathogen smuggles it along a natural reaction pathway. In another strategy the pathogen binds to carbohydrates on glycosylated integrins rather than the Arg-Gly-Asp (RGD) motif to evade triggering the oxidative burst of phagocytes (Hoepelman, 1992). *B. pertussis* uses a type of mimicry by expressing the RGD motif in its filamentous hemagglutinin which then binds to the CR3 receptor on endothelial cells and macrophages. *C. albicans* uses a type of reverse mimicry by expressing the integrin analog, iC3b receptor, to promote interactions with endothelia and perhaps affecting cytoskeletal communication within the endothelia (Rozdzinski et al, 1995). It could also be masquerading in a veneer of iC3b, or competing with neutrophils for the iC3b and inhibiting phagocytosis (Gilmore et al, 1988). The synthesis of C3 by endothelial cells is documented (Brooimans et al, 1990) and the C3 fragments could function as a bridge between *Candida* and neutrophils or *Candida* and the endothelium (Mark, 1989). These same complement bridges could clump the yeast, making it less accessible to phagocytosis (Heidenrich, 1985, Hostetter et al, 1982; Hostetter, 1994).

When iC3b binds to *C. albicans*, the domain recognized by the neutrophilic CR3 is therefore hidden (Gilmore et al, 1988), removing it from interaction with the neutrophil. This competition for iC3b could inhibit the phagocytosis of *C. albicans* by camouflaging the yeast or by simply 'hiding the flag' from the neutrophil. Candida's complement receptors may remove opsonins necessary for phagocytosis by acting as cofactors in the enzymatic degradation of bound complement (Edwards

et al, 1986). One study has demonstrated that *C. albicans* scavenges iron from rosetted erythrocytes (Moors et al, 1992), bypassing the iron-sequestering mechanisms present in human serum that usually restrict candidal growth (Caroline et al, 1964; Esterly et al, 1967; Elin and Wolff, 1973).

It has been observed that erythrocytes are opsonized as bystanders when *Candida albicans* activates complement during an infection and that *C. albicans* rosettes these opsonized erythrocytes, perhaps to acquire scarce iron from their hemoglobin (Moors et al, 1992). The growth of *C. albicans* is dramatically inhibited in the presence of transferrin and growth is restored most efficiently with hemoglobin as compared to ferritin or hemin (Yan et al, 1996). There is also evidence that *C. albicans* produces a hemolytic factor (Manns et al, 1994; Kuprowski, 1966; Salvin, 1952) but this is not widely accepted. A recent study has shown that hemoglobin in defined medium specifically enhances *C. albicans'* ability to bind fibronectin and cultured endothelial cells. Iron-containing proteins, porphyrins and inorganic iron salts were unable to produce this enhancement (Yan et al, 1996). Although the exact mechanism for this induction of fibronectin binding is unknown, altered gene expression and signal transduction in *C. albicans* after adherence to epithelial cells has recently been demonstrated (Bailey, 1995). Fibronectin receptors have been demonstrated in *Candida albicans* with antibodies to $\alpha_5\beta_1$ (CR2) and GRGDSP peptides have been shown to inhibit *C. albicans* adhesion to solid-phase fibronectin (Negre et al, 1994; Santoni et al, 1994). It may be that one of the functions of the CR3-analog on *C. albicans* is to help obtain

nutrients from opsonized erythrocytes for increased growth, increased expression of fibronectin-binding adhesins and consequent increased adherence.

There are several possible mechanisms that the iC3b-receptor of *Candida albicans* may use to contribute to virulence. That it is involved in virulence is supported by the correlation between expression and candidal virulence, the role of the integrin-analog in candidal attachment to endothelial and epithelial surfaces, and the correlation between expression and inhibition of phagocytosis (Csato et al, 1986).

Mycelium Production

Mycelium and germ tube production have been proposed as contributing to virulence but like so many of the other putative factors there are contradictory studies (Ghannoum and Elteen, 1990a; Odds, 1988a,b). Some authors assert that a relationship exists between mycelium production and infection after observing filamentous forms of *Candida albicans* in scrapings from lesions (Odds, 1988a,b; Louria and Grayton, 1964; Saltarelli et al, 1975), assuming hyphae penetrate tissues more readily than yeast cells, form emboli (which lodge in tissue capillaries) and are less ingestible by phagocytes (Odds, 1988a,b). Other researchers claim that the yeast forms are more virulent than the mycelial (Evans, 1980; Simonetti, 1973) and still others saw no difference in infectivity (O'Grady, 1967).

More recent work by Shepard (1985) used morphological mutants of *Candida albicans* that indicated both yeast and mycelial forms are capable of invading soft tissue to cause systemic infection and death in mice. In a rat vaginitis model, where

pseudoestrus is induced with injected estradiol in oophorectomized female rats, production of a mild infection required a higher inoculum of a variant *Candida albicans* strain that fails to germinate *in vivo* as compared to the wild-type strain (Sobel et al, 1984, 1985). This lead the researcher to suggest that hyphae production is important but not essential. Another group, using the rat vaginitis model, studied the vaginopathic potential and intravaginal morphology of a nongerminative *Candida albicans* variant. Although low virulence was demonstrated in systemic infections, this strain was capable of causing a vaginal infection of similar extent and duration as infections induced with germ-tube forming strains of *C. albicans*. This variant strain was unable to produce germ tubes *in vitro* using standard methods but by day 7 of vaginal infection had produced long threads indistinguishable from hyphal filaments (De Bernardis et al, 1993). The different pathogenicity potentials of this strain in different disease models illustrates the problem in studying virulence factors in general and in *Candida albicans* in particular.

Perhaps part of the cause of these discrepancies is the use of different animal models (rabbits, rats, mice), different routes of infection (vaginal, intraperitoneal, intravenous) and different thresholds for "infection" (candidemia, death, colonization of vaginal epithelium). Different tissues and modes of infection will confront the organism with varied environments, perhaps eliciting different responses from *Candida*.

It has also been suggested that the early stages of infection involve the yeast

forms adhering and invading the epithelial surfaces (Klotz et al, 1983). This form resists the intracellular killing of phagocytes (Richardson and Smith, 1981b) and can then develop germ tubes, which biochemically or mechanically pierce the phagocytic cells (Cockayne and Odds, 1985; Louria and Grayton, 1964). Yeast may also disrupt mechanisms within the phagocyte by secreting hydrolases toxic to phagocytic cells (Borg and Rüchel, 1990). Escaping and disseminating to other tissues, the germ tubes are more susceptible to phagocytic killing by attachment of neturophils and induce a respiratory burst and degranulation (Diamond et al, 1978; Diamond et al, 1980; Lyman et al, 1987). Neutrophils have also been shown to have microbiostatic activity as well, inhibiting pseudohyphal growth in abscesses (Sohnle and Hahn, 1993). *Candida albicans* may then convert to the more resistant blastospore. This may explain why yeast and hyphal forms are found in different experimental systems together and separately (Ghannoum and Elteen, 1990a). Killing efficiency of ingested yeast also varies with the phagocytic cell type and resistance of the *Candida* strain to phagocytosis, adding more variables to the picture.

Comparison of virulence between yeasts and hyphal forms is complicated by the difficulty in preparing comparable inocula (Evans et al, 1975). Homogeneous cell suspensions of yeasts are easily obtained from solid or broth culture since yeast growth results from enlargement of a mother cell and formation of blastoconidia (buds) and then a daughter cell. Extension of germ tubes from the original yeast mother cell develop into hydrophobic, adhesive hyphae that form clumps in

nonhomogeneous suspensions (Tronchin et al, 1989). Each filament is divided by septa into several cells, each cell capable of infection. Counting colony forming units does not compensate for this heterogeneous suspension, since these aggregations may disassociate *in vivo*, yielding more infective units (Cutler, 1991).

Various methods can be used to induce the two forms, and studies focused on comparisons of pathogenicity have usually not controlled for the effects the induction method has on virulence. Induction conditions usually involve shifting the nutritional environment and keeping the temperature constant (ie. inoculating yeasts cells into serum and reincubating at 37°C) or keeping the nutritional environment constant and changing the temperature of incubation (ie. protein in the media, temperature from 25°C to 37°C) or varying both temperature and nutritional environment (from 25°C to 37°C and from Sabouraud plates to serum) (Odds, 1988a,b). For example, yeasts grown at 25°C have different surface properties and are more virulent than yeast grown in the same medium at 37°C (Antley and Nester, 1988; Hazen and Hazen, 1987). If suitable morphological mutants could be developed that were deficient only in germ tube production, some of these difficulties could be circumvented. One such mutant, defective in the gene PHR1, has been suggested (Saporito-Irwin et al, 1995).

Adding to the variables in studying the dimorphism of *Candida albicans*, is the development of chains and branches of elongated blastoconidia called pseudohyphae. These heterogenous structures may be so long as to be confused with septate hyphae or simply short elongated blastoconidia. Where germ tubes

develop from yeast cells and have parallel sides with true septa, pseudohyphae may develop from blastoconidia or from a septum in the germ tube and they do not have parallel sides, pinching slightly at the ends. Adhesive characteristics vary in pseudohyphae also, with the same strain demonstrating different characteristics even under identical experimental conditions (Ryley and McGregor, 1986; Shepard, 1985).

When hydrophilic yeast cells are grown at 37°C and are then induced to germinate, a change to hydrophobicity precedes germination (Hazen and Hazen, 1988). It follows that hydrophobic yeasts germinate more quickly than hydrophilic yeasts, are more virulent in mice infected by intravenous infusion, and target different parts of organs when compared to hydrophilic yeasts (Antley, 1988). These studies overcome the morphology of inoculum problem by using homogeneous suspensions of two forms of yeasts.

Expression of various cell surface antigens between the various forms of *Candida albicans* may affect its virulence also. Recent data suggests that mobility of cell-wall components is required for germination (Cassanova, 1990) but it has yet to be shown whether this has any bearing upon adherence.

Coordinated changes in the physical and enzymatic processes of hyphae have been shown during the invasion of host epithelial cells (Rajasingham, 1987). But many molecules and receptors for host proteins that were initially thought to be exclusively produced by hypha have recently been demonstrated in yeasts also. Independent of germination, yeast phase organisms have adhered to and

penetrated the endothelium of whole vascular strips in an *in vitro* model (Klotz et al, 1983). The CR2-like mannoprotein is expressed in yeast and hyphal cell walls and surfaces (Kanbe et al, 1991) and certain nutritional factors induce adhesins in yeast forms (Critchley and Douglas, 1987; McCourtie and Douglas, 1985a).

Recently, Swoboda has applied molecular techniques to the study of morphogenesis by looking at the mRNA of a putative ribosomal protein and of four glycolytic enzymes. Although transient changes in mRNA were seen between the two phases, they were more associated with the physiological state than the alterations in cell shape (Swoboda et al, 1994; Swoboda et al, 1995).

Studies in hyphal versus yeast virulence would be accelerated if agents were developed that specifically inhibit germination without affecting yeast (or host) viability. Drugs, such as the azoles, can suppress germination (Odds, 1988b) and have been used in hyphal studies (Ryley and Ryley, 1990) but since their effects are not limited to inhibition of candidal morphogenesis, conclusions drawn from these studies are limited. The Fab antibody preparation used by Casanova (1990) to restrict cell-wall component mobility during germination, and thereby suppressing germination, is another candidate that does not completely block germination. Any proposed germination inhibitor must not influence the host, must be able to reach the fungal cells *in vivo*, and its effect must be complete and specific for germination (Cutler, 1991). Gamma interferon inhibited germination of *Candida albicans* by 35-50% in studies by Kalo-Klein (1990), suggesting one part of the *in vivo* mechanism for elimination of the organism.

Phospholipase

Membrane-damaging phospholipase enzyme is a putative virulence factor of *Candida albicans* associated with mycelium penetration of host tissue (Salyers and Whitt, 1994; Pugh and Cawson, 1975; Ghannoum and Elteen, 1990a). Phospholipases contribute to the pathogenicity of bacteria (Wright et al, 1990b; Titball et al, 1989; Marques et al, 1989), rickettsiae (Silverman, 1992), and protozoa (Saffer et al, 1989) by contributing to the injury, invasion or exit from various host cells (Saffer et al, 1989; Silverman, 1992; Wright et al, 1990b). Phospholipases C and A_2 are suspected of removing processed antigens from the surface of antigen-presenting cells (Falo et al, 1986; Falo et al, 1987). Thus, it is probable that extracellular phospholipases secreted by *Candida albicans* enhance pathogenicity.

Phospholipase activity has been found to be associated with bud formation in rapidly growing cultures of *Candida albicans*, as well as at the periphery of the cell and secreted into the medium (Pugh, 1975). Experiments with chick chorio-allantoic membrane (CAM) showed after six hours that many blastospores had intense enzyme activity over the entire cell surface (with subsequent autolysis and localized damage to the CAM) while hyphae had a slight concentration of activity towards the growing tip (Pugh, 1977). Enzyme activity in the older portions of the hyphae was intracellular and involved with cellular metabolism and eventual autolysis (Pugh, 1977).

The types of enzymes secreted by *C. albicans* include phospholipase A (Barret-Bee et al, 1959), phospholipase B (Banno et al, 1985), phospholipase C (Pugh,

and Cawson, 1977), lysophospholipase and lysophospholipase-transacylase (Banno et al, 1985; Takahashian et al, 1991). If some studies with *Saccharomyces cerevisiae* hold true for *C. albicans* also, some of these different phospholipase activities may be contained within the same enzyme (Lee et al, 1994). More recent studies indicate that phospholipase B and lysophospholipase-transacylase are the main phospholipases secreted by *C. albicans* (Ibrahim et al, 1995).

C. albicans is the only *Candida* species known to secrete phospholipases (Samaranayake et al, 1984; Filler et al, 1994), although a large variation in phospholipase activity between different isolates of *C. albicans* has been found (Price and Cawson, 1977; Samaranayake et al, 1984). In a recent study, isolates from blood secreted more phospholipases and formed more germ tubes than commensal strains did (Ibrahim et al, 1995). This same phenomenon is seen with pathogenic bacteria such as clinical isolates of *Pseudomonas aeruginosa* which also constitutively express a variety of virulence factors at a higher level than that expressed by commensal isolates (Janda, 1981).

In the infant mouse model of hematogenous invasion from the colonized gut, an isolate with high extracellular phospholipase activity (as measured by the egg yolk agar plate method (Samaranayake et al, 1984)) was invasive whereas the isolate with low extracellular phospholipase activity was not (Ibrahim et al, 1995). The noninvasive strain was still able to colonize the gastric mucosa, indicating that secretion of phospholipases is not required for mucosal colonization (Ibrahim et al, 1995). Other putative virulence factors of these two strains were compared and no

correlation was found between extracellular phospholipase or proteinase activity and endothelial cell damage, as measured by a modified ^{51}Cr release assay (Ibrahim et al, 1993), and germination rates were essentially the same (Ibrahim et al, 1995). This is not surprising for the proteinase since its optimum activity pH is 2.2-4.5 and the pH of the cell damage assay is presumed to be neutral to slightly alkaline because it uses endothelial cell monolayer cultures (Remold et al, 1968; Rüchel, 1981; Ibrahim et al, 1995). Lysophopholipase and phospholipase have activity in a broader pH range (pH 3-7) (Barrett-Bee, 1985). However, it has been shown that with agar-grown *C. albicans*, phospholipase production takes place only within a limited pH range of 3.6-4.7 (Samaranayake et al, 1984). What is indicated by all of this is that at the relatively neutral pH of in vivo endothelium some factors other than phospholipase and acid proteinase are responsible for endothelial cell damage (Ibrahim et al, 1993; Filler et al, 1994; Ghannoum el al, 1992).

Studies with *C. albicans* have shown a positive correlation between extracellular phospholipase activity, adhesion to buccal epithelial cells, and mortality in the iv mouse model (Barrett-Bee, 1985). However, similar studies with endothelial cells indicated no correlation between extracellular phospholipase production and endothelial cell adherence (Ibrahim et al, 1995). All of the isolates in the latter studies showed a propensity for adherence to endothelial cell monolayers regardless of their in vitro phospholipase activity, so that the contribution to adherence by the phospholipase enzyme activity is not immediately evident (Ibrahim et al, 1995).

Extracellular phospholipases contribute to virulence in bacteria by degrading the components of the host cellular membrane or by changing their surface characteristics, facilitating penetration and adherence by the pathogen (Silverman et al, 1992; Walker et al, 1983; Winkler and Miller, 1980; Saffer et al, 1989). Ultrastructural studies of epithelial invasion by *C. albicans* show penetration of the epithelial cell by *C. albicans* (Marrie and Costeron, 1981). This fusion may occur in an acidic microenvironment and may be facilitated by candidal phospholipases. There is some evidence that high phospholipase production correlates with increased adherence to buccal epithelial cells (Barrett-Bee, 1985) and therefore may contribute to oral mucocutaneous infections. But since this correlation was not seen when endothelial cell adherence was examined with phospholipase production, and gut colonization was not compromised by lower phospholipase activity, it appears phospholipase activity is not necessary for endothelial cell adherence or hematogenous invasion (Ibrahim et al, 1995). However, if candidal infection is due to a sum of various factors from *Candida albicans*, balanced against host factors at the host site, extracellular phospholipase activity secreted by *C. albicans* may contribute to virulence in certain niches. To characterize the contribution of phospholipase to virulence, isogeneic strain pairs that differ only in the production of extracellular phospholipase activity need to be constructed. The relative levels of virulence of these strains should then be compared in various animal models which mimic human infections as much as possible.

Acid Proteinase Secretion

The 'classic' candidal secreted acid (aspartyl) proteinase (or SAP, nomenclature agreed upon at the 1993 American Society for Microbiology Meeting on Candida and Candidiasis) has properties similar to pepsin and cathepsin-D, being inhibited by equimolar pepstatin (Rüchel, 1981) and partially inhibited by chymostatin and by serum protease inhibitors, β2-macroglobulin, and α-1-antitrypsin. Described as a single polypeptide chain mannoprotein with a molecular mass of 40 - 45kd, the optimal activity of this enzyme is from pH 2.2 - 4.5 with some activity reported up to pH 5.5. Induced by protein and repressed by amino acids and ammonium salts (Bannerjee et al, 1991), SAP is irreversibly denatured at pH 7.5 (Remold et al, 1968; Rüchel, 1981; Rüchel, 1982; Negi et al, 1984; Ray and Payne, 1990; Ray et al, 1991; White and Agabian, 1995). Substrates which this enzyme degrades include BSA, keratin, hemoglobin, casein, IgA (heavy chains), denatured collagen, transferrin, and salivary proteins (Rüchel, 1981; Rüchel, 1982; Rüchel, 1986; Negi et al, 1984; Hatori et al, 1984; Ray and Payne; 1990). Some studies have indicated that enzyme activity is three times higher with bovine hemoglobin than with BSA, which is used in most assays (Negi et al, 1984). It has also been speculated that proteases secreted by *C. albicans* may degrade cytokines secreted by *C. albicans* - damaged endothelial cells, reducing the accumulation of cytokines (Filler et al, 1996).

Candida albicans and some other *Candida* species secrete proteinases when they are grown in media containing proteins as sole nitrogen sources (MacDonald

and Odds, 1980; Rüchel, 1981; Rüchel et al, 1982). Evidence that secretory proteolytic activity is an important determinant of pathogenicity is derived from the following avenues:

1) Aspartyl or acid proteinase is secreted only by the *Candida* species which cause disease most often, with *C. albicans* isolates strongly proteolytic, followed by *C. tropicalis* and *C. parapsilosis* isolates which are proteolytic to a lesser extent (MacDonald, 1984; Rüchel et al, 1983). The other *Candida* species of clinical relevance like *C. glabrata, C. krusei, C. kefyr* and *C. guilliermondii* do not produce extracellular proteinases (MacDonald, 1984; Rüchel et al, 1983) This mirrors the prevalence of infection in humans. *Candida albicans* strains isolated from women symptomatic of vaginitis produced a higher level of secretion when compared to strains isolated from asymptomatic women (Cassone et al, 1987). In a study of *Candida albicans* strains, highly vaginopathic strains showed early *in vivo* expression of SAP 1 and SAP2 mRNA (SAP: secreted aspartyl proteinase genes) and also adhered more readily to vaginal epithelial cells as compared to nonvaginopathic strains, suggesting that acid proteinase may contribute to adherence or be coordinately regulated with adherence attributes (De Bernardis et al, 1995).

2) In chemically induced mutants of *C. albicans*, proteinase deficiency was linked to lower virulence in mice infected iv (Kwon-Chung et al, 1985; MacDonald and Odds, 1983; Ross et al, 1990).

3) This aspartic protease degrades keratin and collagen (Negi et al, 1984; Ray

and Payne, 1990; Hattori, 1984), constituents of the epidermis and dermis. Its activity is associated with tissue invasion in experimental skin infections (Ray and Payne, 1994) and it is visualized at the surface of the fungus found in infected tissue by indirect immunofluorescence (Rüchel et al, 1983; Rüchel et al, 1991). Inoculation of *C.albicans* into the chorioallantoic membrane of embryonated chicken eggs also demonstrated by immunohistochemical methods secreted proteinase on the surface of the yeast cells before invasion of the tissue. Invasion and mortality were also correlated with the level of in vitro proteinase activity (Shimizu et al, 1987). This proteinase is expressed by *Candida albicans* after attachment of yeast cells to host epithelium *in vitro* and *in vivo* (Borg and Rüchel, 1988). Cavitations in keratin-rich corneocytes are only found around aspartyl proteinase secreting species and are blocked by pepstatin, a proteinase inhibitor (Ray et al, 1984).

4) IgG antibody titers to aspartic proteinase are found in the sera of patients with invasive or disseminated candidiasis (MacDonald and Odds, 1980; Ray and Payne, 1987; Rüchel et al, 1988). Although ELISA has been used to detect the *Candida* aspartyl (acid) proteinase in the serum of these patients (Rüchel et al, 1988), its contribution to virulence in the bloodstream cannot be determined because the pH in this environment (7.2 - 7.5) is far above optimal (pH 3.2-5.5) for acid proteinase activity. In the rat vaginitis model, acquired anticandidal protection was mediated by antibodies to mannan and secreted aspartyl proteinase and protection was tranferrable (Cassone et al, 1995).

5) Aspartic proteinase is found in the vaginal secretions of patients with vaginitis and not found in the secretions of asymptomatic women (De Bernardis et al, 1990).

6) The expression of fungal aspartic proteinase has been demonstrated in murine macrophages after phagocytosis of *C. albicans* (Borg and Rüchel, 1990). This expression has been linked to increased resistance to killing after phagocytosis (MacDonald and Odds, 1983).

7) *C. albicans'* aspartyl proteinase was found to enhance vascular permeability by activating the plasma kinin generating system, producing the vasoactive bradykinin in pharmacological amounts during *in vitro* and *in vivo* assays (Kaminishi, 1990).

8) The acid protease inhibitor pepstatin blocks *C. albicans'* adherence to cultured human epidermal keratinocytes (Ollert et al, 1993) and oral epithelium (Borg and Rüchel, 1988) and blocks events such as cavitation in the early invasion of murine skin (Ray and Payne, 1988). Pepstatin has also modified the course of experimental systemic candidiasis in mice (Rüchel et al, 1990).

Another study found inducible extracellular proteinase produced by both a low-virulence mutant of *Candida albicans* and its wild-type parent. The mutant grew more slowly in a high protein media, so that the threshold level of proteinase which may be required *in vivo* to facilitate pathogenicity, as suggested by Kwon-Chung et al, was not reached and hence the reduced virulence (Edison, 1988; Kwon-Chung et al, 1985).

The experimental model for virulence used in an experiment must also be appraised, as became evident in work with a nongerminative, low proteinase producing variant of *C. albicans* which expressed low virulence in a systemic mouse model and elevated vaginopathic potential in the rat vaginitis model (De Bernardis et al, 1993; Kondoh et al, 1987).

Candida albicans produces secreted and intracellular proteinases (Staib, 1965; Staib, 1969; Portillo et al, 1986), though the secreted aspartyl acid proteinase forms are the most studied and are regarded as virulence factors. Although at first considered to be just one protein, discrepancies appeared concerning molecular weight, pI, N-terminal sequence of the protein, inducing conditions, substrate specificity, and glycosylation. This is finally being sorted out through cloning and expression studies on the seven identified genes: SAP1 (Hube et al, 1991), SAP2 (Wright et al, 1992), SAP3 (White et al, 1993), SAP4 (Miyasaki et al, 1994), SAP5, SAP6, SAP7 (Monod et al, 1994). The gene PEP1, shown to be regulated by white-opaque switching, is thought to actually be SAP1 (Morrow et al, 1992). SAP1 has been mapped to chromosome 6 and SAP2 to chromosome R using contour-clamped homogeneous electric field chromosome separations and high stringency hybridization with several strains of *C. albicans* (Magee et al, 1993).

Secreted aspartyl proteinase activity may be restricted to host sites with a local drop in pH (Germaine and Tellefson, 1981). Sites where neutrophil glycolysis is active in acute inflammatory reactions or on the acidic skin surface are likely candidates. SAP not only requires an acidic pH for activity but several laboratories

have shown a requirement for an acidic pH in the inducing media as well (White and Agabian, 1995; Homma et al 1993; Matsuda, 1986; Germain, 1981;) while others have questioned a direct effect (Homma et al, 1993). The expression of six different SAP genes from different strains and cell types was examined, under varying environmental conditions, including various pH levels (White and Agabian, 1995; Hube et al, 1994). Probes for SAP4, SAP5 and SAP6 hybridized with mRNA from cells producing hyphae in serum at neutral pH (White and Agabian, 1995; Hube et al, 1994) while the highest levels of SAP1 occurred at pH 5.5, of Sap 2 at pH 4.1 and of Sap 3 at pH 3.2, in the cells and strains that expressed them at all. Although most strains express high levels of SAP2 mRNA and low levels of SAP1 mRNA in standard proteinase-inducing media, there are exceptions. SAP1 appears to be constitutively expressed in opaque cells from the white/opaque phenotypic switching strain WO-1 (Wright et al, 1992). White concluded that while the pH of the medium does not affect the pattern of SAP gene expression, which is determined by the strain and cell type, it does affect the levels of Sap isoenzymes. This work also demonstrated that RNA and protein levels are comparable under most conditions suggesting that little post-transcriptional regulation occurs (White and Agabian, 1995).

When the expression of seven SAP genes was examined by Hube et al., SAP2 was found to be the most abundant transcript in the yeast form of the fungus under a wide rage of conditions and which is repressed by amino acids and NH_4^+ at pH 4.0 (Hube et al, 1994). Most published biochemical studies of partially purified *C.*

albicans secretory proteinase are likely to reflect characterizations of the SAP2 gene product. Alignments of the N-terminal amino acid sequences of the various *C. albicans* proteinases purified by different groups support this view (Ray and Payne, 1991; Yamamoto et al, 1992; Rüchel et al, 1992; Hube et al, 1991; Ganesan et al, 1991; Morrison et al, 1993; Togni, 1991; Wright, et al, 1992). Some type of carbon source is needed for SAP2 expression, presumably this reflects the need for a carbon and energy source for growth and/or Sap2-proteinase production. SAP2 expression was only observed during growth, indicating that energy may be required for transport of a Sap2-proteinase inducer into the cell, for signal transduction via a membrane receptor or for proteinase biosynthesis and secretion (Hube et al, 1994). Although amino acids repress SAP2 expression, peptides rather than the intact BSA protein seem to be the inducers . This study found that pH of the medium had an indirect effect on SAP2 expression by influencing the rate at which inducing peptides are generated from BSA by Sap2 activity (Hube et al, 1994). Their model of induction suggests that a basal level of proteinase expression may occur under all culture conditions due to the expression of one or more SAP genes. A positive feedback mechanism involving peptides from the proteolysis of high-molecular-weight protein appears to lead to Sap2-proteinase expression. Since SAP2 mRNA was induced poorly at pH 2.5 and at pH 6.5 even in the presence of peptides, a failure of a peptide-transport system or a signal-transduction pathway to operate at neutral or relatively low pH value may account for this phenomenon (Hube et al, 1994).

While transcripts of SAP4-SAP6 were found during serum-induced germ-tube formation, it was not coupled obligately to hyphal production as these genes were not expressed in hyphal cells that were induced by the pH/temperature-regulated transition. Amino acids did not down-regulate the expression of SAP4-6 and a pH of 6.0 or higher was necessary to obtain high SAP4-6 mRNA levels (Hube et al, 1994). It is not clear whether these enzymes are active under these conditions since most aspartyl (acid) proteinases have a pH optimum lower that 7.0 (Davis, 1990; Tang and Wong, 1987). One possible scenario is that SAP4-6 are expressed during hyphal transition in vivo, but the gene products are only active in acidic microenvironments or that they have a non-enzymatic role in a process such as cell adhesion or cell remodeling (Cutler, 1991).

Polyclonal rabbit antiserum prepared against Sap2 reacts with SAP1, Sap2, and Sap3 because of the high degree of homology (Monod et al, 1994) and Sap2 is probably the 43kd protein Homma et al (1992) detected with a similarly prepared antiserum. Intracellular proteinases have been detected and of the three proteinases associated with intracellular compartments, only one is similar to the secreted aspartyl acid proteinase, with a pH optima about 5.0, size of 60-kD, and inhibited by pepstatin. The other intracellular proteinases are a dipeptidase and an aminopeptidase (Portillo et al, 1986). Lott has cloned and sequenced an aspartyl proteinase from *C. albicans* that is not secreted and appears more closely related to the *Saccharomyces cerevisiae* PrA gene, whose product is a vacuolar protein (Lott et al, 1989), than to a SAP. Intracellular pools of the secreted aspartyl

proteinase have been located (Ross et al, 1990) and two immunologically detected proteins have been shown to be precursor (45kd) and secreted (43kd) forms of the aspartyl proteinase (although specific activity was not demonstrated) (Homma et al, 1992; Homma et al, 1993). Two other intracellular proteinases have pH optima of 7.5 and 8.0 and may aid pathogenesis if yeast cells are disrupted at tissue sites with near neutral pH (Portillo et al, 1986; Logan, 1986).

Toxins

The histopathology and symptomatology found in systemic mycoses, as well as in experimental fungus infections, suggests the production of toxins in the invaded tissues (Salvin, 1952; Hasenclever and Mitchell, 1962; Isenberg et al, 1963; Iwata, 1977). Clinical evidence derived from patients with systemic candidiasis indicates that they occasionally manifested a clinical syndrome indistinguishable from Gram-negative sepsis (Louria, 1985). Work to characterize the toxin(s) has divided the substances into high-molecular weight and low-molecular weight toxins. Like bacterial exotoxins, these fungal toxins are highly antigenic and can easily be converted to toxoid with formalin.

High molecular Weight Toxins: Glycoprotein toxins and Canditoxin

Toxic substances containing carbohydrates as toxic components have been found in fungal cell walls, intact cells, cytoplasmic extract, and from the growth media of virulent strains (Masler et al, 1966; Cutler et al, 1972; Iwata et al, 1967a; Iwata and Uchida, 1967b,c,d; Iwata, 1975). Coarse extracts (Cutler et al, 1972) as well as purified fractions (Iwata, 1975) exhibited potent and acute toxicity for mice.

Although pyrogenic in rabbits, the cell walls and intact cells did not coagulate a haemolysate of horseshoe crabs, a test for the presence of the bacterial endotoxin (Cutler et al, 1972). Examination of the gross chemical constituents of fungal substances that exhibited toxic effects has demonstrated that they differ substantially from those of classical bacterial endotoxin and they also fail to be active in several experimental systems where bacterial endotoxin is strongly active (Cutler et al, 1972).

Characterization of these glycoprotein toxins suggests that both protein and sugar (usually mannose or glucose) moieties are necessary for the toxic activity. Cutler et al isolated two toxic fractions which differed in the ratio of carbohydrate to protein and in the toxic effect on mice and rabbits (Cutler et al, 1972). The extract which was toxic only to mice and apyrogenic to rabbits also had relatively low amounts of protein whereas the fraction which was toxic to both species had relatively high amounts of protein (Cutler et al, 1972). It is hypothesized that the carbohydrate moiety causes toxicity in mice and the proportion of protein determines whether the substance is pyrogenic.

The lethal effect of Iwata's toxin (MW of 50kD) was lost after heating at 56°C for 30 minutes and no hemolysis was demonstrated with human, mouse or rabbit erythrocytes. This toxin also heightened an experimental mouse infection using the strain of *C. albicans* from which the toxin was isolated (Iwata et al, 1967a).

Clearly, both teams of researchers were working with glycoproteins with toxic effects in animal models. Since they used very different purification procedures, it

is unknown whether they were dealing with the same entities. Protein electrophoresis and immunoblots would reveal more about their similarities and dissimilarities. It is obvious that these toxic fractions from *C. albicans* have only a few functional characteristics similar to bacterial endotoxin, which we now know is a lipopolysaccharide. The mannoproteins of *C. albicans* have been implicated as adhesins involved in colonization (Al-Bassam et al, 1985; Elorza et al 1985). Yeast mannans have also been reported to have the toxic capacity to initiate fatal anaphylaxis-like reactions (Kind et al, 1972; Kind et al, 1970).

Canditoxin, though extensively studied by one laboratory, is very unusual in that it is uniquely produced by a single isolate of *Candida albicans* from a patient with *Candida* meningitis (Iwata et al, 1967a; Odds, 1988a). Other researchers could not find similar substances in a survey of *Candida albicans* strains (Chattaway et al, 1971).

Purification of this substance from yeast cytoplasmic extract revealed it to be a simple acidic protein with a molecular weight of 75kD. Its amino acid composition shows some homology to certain bacterial toxins such as secreted by *Clostridium* sp. and *Staphylococcus aureus*. It is inactivated by heating to 50°C for 30 minutes and is composed of four subunits, as determined by enzymatic and physicochemical studies. It is acutely toxic for mice with a LD_{50} by intravenous injection of 0.3µg/g body weight. The treated animals died within 48 hours from an anaphylaxis-like reaction (Iwata et al, 1967a; Iwata and Uchida, 1968). Canditoxin is pharmacologically active when tested in the isolated toad heart and guinea pig

intestine models. Although it did not demonstrate any enzymatic activity typical of lipase, phospholipases, cholinesterase, α-amylase, proteinase or aminopeptidases, it did exert a marked cytopathic effect on cultured cells of the Hep#2 and FM 3A strains (Iwata and Uchida, 1968).

Production in vivo of canditoxin by *C. albicans* was demonstrated by fluorescent antibody staining of mouse tissues infected intravenously with *C. albicans*. Infection of mice with a canditoxin-producing strain was enhanced by the simultaneous administration of a sublethal dose of the purified toxin. There was more extensive proliferation of the yeast with more severe histopathological changes (Iwata, 1975).

Canditoxin's potent antigenicity was evidenced by the high titer of antibody, present in the IgG, IgA, and IgM fractions. This was demonstrated by agglutination and precipitin reactions in serum of rabbits which had been administered canditoxin-toxoid subcutaneously. The IgM fraction was the most effective in suppressing mouse infection with the canditoxin-producing strain of *C. albicans* (Iwata et al, 1967a; Iwata and Uchida, 1967c; Iwata and Uchida, 1968; Iwata, 1975).

Other researchers have purified a fraction similar to that from the Japanese strain and identified it to be an acid phosphatase, supporting the possibility that hydrolytic enzymes may be responsible for the observed toxic effects (Chattaway et al, 1971). No corroboration of the canditoxin has appeared from any other laboratory (Odds, 1988a).

More recent studies found that rats injected with lyophilized cellular supernatant or live, whole cells of a clinical isolate of *C. albicans* had higher serum levels of acid

phosphatase than saline injected controls, indicating lysosomal damage and spillage into the blood. The treated rats also showed significant changes in DNA activity in renal and hepatic cells, as measured by a modified Feulgen nuclear reaction (Yemma and Berk, 1994). If indeed the canditoxin is an acid phosphatase, these researchers did try to distinguish between the toxin from *C. albicans* and that from lysosomal sources.

Low Molecular Weight Toxins:

During the course of purifying canditoxin, researchers isolated six different low-molecular weight toxins that shared shock-evoking and/or lethal activities. Only one was characterized, with a molecular weight of 164 and LD_{50} for mice 5µg/g body weight after intravenous injection (Iwata, 1975).

Another toxin in this group is the immunosuppressive gliotoxin (Shah and Larsen, 1989; Shah et al, 1990, 1992). With a molecular weight of 324 kDa, it contains disulfide bonds which are important to its activity. Its antiphagocytic properties are demonstrated in leukocyte inhibition studies (Shah and Larsen, 1992).

Toxins have been examined 1) for their infection-enhancing activities, 2) the infection-suppressing activity of antisera or immunoglobulin which reacts with the toxin, 3) for in vivo production during the course of infection by the strain producing it, 4) for the influence on host defense mechanisms. The only on-going work appears to be with gliotoxin and its effects on the host immune system (Liu, personal communication).

It is unlikely that a microbe that is usually a benign, commensal organism would secrete a potently bioactive toxin and still be an opportunistic pathogen. The pathological changes demonstrated around tissue foci of *Candida* infections may be the result of host inflammatory responses to *Candida* and its cell wall mannans or other putative virulence factors. If *Candida albicans* does secrete molecules with biological potency, they may be tightly regulated by environmental signals and be strain specific.

Heat-shock Protection

Heat-shock proteins have been proposed as virulence factors in bacteria and fungi. Heat-shock proteins in bacteria are dramatically upregulated following temperature elevation, such as bacteria experience when they enter a mammalian host (Lathigra, 1991; Neidhardt and VanBogelen, 1987). Studies also document virulence factors in bacteria coordinately regulated by thermal and osmotic stress through two-component signaling pathways involving phosphorylation and dephosphorylation (DiRita and Mekalanos, 1989; Finlay and Falkow, 1989; Gross et al, 1989). At least one osmosensing signal transduction pathway has been suggested for mammalian cells (Galcheva-Gargova et al, 1994). Temperature regulation of stress proteins and virulence factors as well as the relationship of estrogen to stress survival all seemed to indicate that there could be a causal connection between estrogen exposure and increased survival of *C. albicans* in the human host.

Physiologically in the microbe or mammalian host, hsp90 serves as a chaperone

for unfolded polypeptides, for folding newly translated proteins, for maintaining correct protein folding, and to prevent premature degradation of proteins (Gething and Sambrook, 1992; Pratt, 1994). Heat-shock proteins can also function as proteases to degrade polypeptides that have unfolded as a result of stress (Lipinska, 1989). During the infectious process, the microbe is usually subjected to an increase in temperature and the level of polypeptides in need of chaperone protection or protease activity may be elevated due to altered patterns of protein synthesis or of protein translocation or by the denaturation of proteins (Ellis, 1990; Rothman, 1989).

The role of hsp 90 and other stress proteins as chaperones to the steroid receptors, and the presence of an estrogen-binding protein in *C. albicans,* have already been discussed. Reports of antibodies in the sera of systemic candidiasis patients to a 47kDa fragment of *C. albicans'* hsp90 implicated it as an immunodominant antigen (Matthews and Burnie, 1984, 1989; Matthews et al, 1987). Antigens that elicit antibody formation often are important to the pathogenic process and heat-shock proteins are major targets for specific immunity in many infections (Matthews and Burnie, 1987,1992; Young, 1992; Morrison et al, 1989). Protection from different microbes may occur because of the cross-reactivity to shared epitopes on highly conserved heat-shock proteins like hsp90 (Kaufman, 1990). The epitope recognized by all patients with antibody to the 47 kDa antigen is highly conserved and proposed as the protein-binding site of human hsp 90 (Matthews and Burnie, 1989; Schwarz, 1991; Sullivan and Toft, 1993). A murine monoclonal

antibody raised against this epitope, given prophylactically, reduced mortality in a mouse model of invasive candidiasis (Matthews et al, 1991). All of this points to the importance of the candidal hsp90 to the infectious process.

There are benefits for the host in using such conserved targets for specific protective immunity, as long as the host can avoid, through its own regulatory network, development of autoimmune reactions, such as can be seen in chlamydial infections (Morrison et al, 1989; Young et al, 1988). This cross-protective immunity can be achieved because different microbial pathogens share this antigen (Kaufman, 1990). A high level of specific, high affinity antibody may be available early in an infection if there is repeated exposure to the same epitope on potential pathogens and commensals. The Gram-positive bacterium *Corynebacterium jeikeium* cross-reacts with antibody to the *C. albicans* hsp90 (Matthews, 1991). Mutations to the conserved antigenic epitopes that would make the organism more resistant to host antibodies would probably also be lethal to the organism.

The connection between steroid hormones and cell protection probably stems from the role steroids play in regulating apoptosis as part of embryonic development, sexual differentiation and maturation in mammals (Iwata, 1995). Tissue sizes and shapes are under the influence of steroid hormones via the regulation of metabolism and cell turnover. Glucocorticoid-induced apoptosis is dependent on the binding of glucocorticoids to their receptors (Duval et al, 1984) and under certain conditions, like T-cell hybridomas, glucocorticoids inhibit apoptosis (Sloviter et al, 1993). It has been demonstrated experimentally that

glucocorticoids induce thermotolerance in mammalian cells after a lag of 2-3 hours, which suggests protein synthesis (Fisher et al, 1986). On the other hand heat shock was shown to induce apoptosis in some thymic cells but the surviving cells were protected from glucocorticoid-induced apoptosis (Migliorati et al, 1992). The anti-apoptotic effect induced by thermal stress was correlated with the induction of heat shock proteins and abolished by the inhibition of protein synthesis. Another study found that cellular stress, in the form of serum starvation and heat shock, and glucocorticoid protect fibroblasts from the cytotoxic effects of tumor necrosis factor alpha (TNF-α) (Pagliacci et al, 1993). This protection also required de novo mRNA and protein synthesis. Heat shock protein 90 (hsp90) is an essential accessory protein for glucocorticoid receptors (Bresnick et al, 1989; Picard et al, 1990a) and stabilizes estrogen receptors (Smith and Toft, 1993a). There is some evidence that corticosterone levels in vivo regulate corticosteroid receptor mRNA and the expression of hsp90 (Patchev et al, 1994). These interactions between heat shock proteins and glucocorticoids suggest that they belong to a common evolutionary pathway.

Other steroids affect apoptosis in addition to glucocorticoid. It has been demonstrated in vivo that estrogen inhibits and androgens stimulate ovarian cell apoptosis (Billig et al, 1993). In another study, 17β-estradiol protected MCF-7 (human breast cancer) cells from apoptosis through the bcl-2 pathway (Wang and Phang, 1995). Estradiol has been shown to protect neurons from oxidative stress-induced cell death in vitro, in an estrogen receptor-independent manner (Mydlarski

et al, 1995; Behl et al, 1995). This protection from oxidative stress may be due to the antioxidant properties of 17β-estradiol rather than the induction of stress proteins. Estrogens with phenolic structures demonstrated inhibition of lipid peroxidation (Sugioka et al, 1987). Estrogen has been shown to facilitate the expression of small M(r) heat shock proteins in osteoblasts (Cooper and Uoshima, 1994) and other tissues (Ciocca and Luque, 1991). In chicken oviducts and human breast cancer cells, estrogen induces a transferrin receptor-like membrane protein that can also be induced by heat shock (Poola and Kiang, 1994). Sequence homology of this protein with chicken heat-shock protein (cHsp108) and yeast hsp90 demonstrates the highly conserved nature of heat shock proteins.

Work with the human progesterone receptor (PR) and stress in breast cancer cells has shown that chemical and thermal stress enhanced PR-mediated gene transcription (Edwards et al, 1992). However, increased cellular concentration of hsp90 induced from elevated temperatures interfered with ER-dependent transcription (Sabbah et al, 1996). The differences may be due to the type of cells used as well as peculiarities of the various steroid receptors and their associated proteins

HOST EFFECTS ON *C. ALBICANS* VIRULENCE

Candida albicans commonly colonizes human mucosal epithelium, where it maintains an ecological balance with normal bacterial flora (Savage, 1972, Sobel, 1992; Liljemark, 1973; Narayanan and Rao, 1976). Overgrowth by *Candida* may be suppressed by competition for receptor sites on epithelial cells (Helstrom, 1979).

Changes in physical, chemical or immunological conditions can alter the balance and trigger candidal overgrowth. The physical environment can be affected by trauma to mucosal tissue, phase of menstrual cycle where fluctuating hormone levels affect vaginal epithelial cell characteristics and mucous viscosity, or systemic antibiotics which affect bacterial flora populations (Caruso, 1964; Oriel and Waterworth, 1975; Narayanan and Rao, 1976). Physio-chemical conditions are modulated by factors such as pH, hormones, and blood levels of glucose (Kinsman, 1986). Immunological status, especially as expressed by the mucosal and cellular systems, can be influenced by these same changes and also by immunosuppressive therapy or pathological conditions (such as AIDS) (Rhoads et al, 1987, Witkin, 1983; Hobbs, 1977; Witkin, 1986; Kalo-Klien and Witkin, 1990).

Hormones as Messengers

Chemical communication is as essential for unicellular organisms as it is for multicellular organisms. Although hormones are obviously signaling molecules in the internal communication system of mammals where they induce specific responses, it has become evident that unicellular organisms have analogous systems of chemical communication (Huxley, 1935; Gooday, 1974, 1983; O'Day, 1981; Ende, 1984). The most extensively investigated fungal systems involve endogenous fungal hormones which regulate sexual reproduction between two individuals of the same fungal species (Sakurai et al, 1984).

The α/a mating system of *Saccharomyces cerevisiae* is known in greatest genetic and biochemical detail (Thorner, 1981; Nakagawa and Yanagishima, 1982;

Cross et al, 1988; Herskowitz, 1988; Yoshida et al, 1989; Fields, 1990). The α- and a-factors, encoded by the STE2 and STE3 genes respectively, are each recognized by a specific membrane receptor in the opposite cell-type. The receptors are coupled with the same heterotrimeric G-protein and ligand binding to the appropriate receptor triggers a signal-transduction process (Marsh and Herskowitz, 1988; Marsh, 1991). Cross-reactivity has been demonstrated between α-factors and a-cells among related species of *Saccharomyces* and *Hansenula* (McCullough and Herskowitz, 1979; Yanagishima, 1981; Fujimura, 1982, 1983; Sakuai, 1984; Yoshida et al, 1989).

Research suggests that unicellular and filamentous fungi produce compounds which are similar to mammalian hormones (Bu'Lock, 1976; Martin et al, 1978; Bramley et al, 1991). The characterization of the estrogenic mycotoxin, zearalenone, produced by *Geibberella zeae* and *Fusarium* spp., suggests that it can act as a broad-spectrum regulator of sexual reproduction in fungi (Nelson, 1971; Plascencia, 1990) and can act as an estrogen in mammalian systems (Katzenellenbogen et al, 1979).

Some plant isoflavinoids, like genistein, which regulate gene transcription in symbiotic soil bacteria *Bradyrhizobium japonicum,* also bind to the mammalian estrogen receptor, activate the receptor and stimulate transcription of estrogen-responsive genes (Kossiak et al, 1990; Baker, 1992; Martin et al, 1978). Not only is homology found between bacterial and mammalian dehydrogenases that regulate the concentration of the signal molecules (Agarwal et al, 1989; Peltoketo

et al, 1988; Luu-The et al, 1989; Baker, 1989), bacterial cell extracts also contain small amounts of material that cross react with antibodies to mammalian hormones (Goldstein, 1982; Domingue et al, 1986; Acevedo et al, 1987). These microbial substances have chromatographic elution similarities to the corresponding mammalian hormone and possess similar biological activity, which is removed by antihormone antibody (LeRoith et al, 1981b; LeRoith et al, 1985; LeRoith et al, 1986). Insulin, glucagon and other polypeptide hormones have been the most extensively investigated across species and insulin has been found in every microorganism examined, from archaebacteria to microbial eukaryotes (LeRoith et al, 1985; LeRoith et al, 1981a; Flawia and Torres, 1972,1973). Proteins that bind mammalian hormones, have been found in many microbes, from *Pseudomonas testeroni* to *Saccharomyces cerevisiae* and *Candida albicans* (Watanabe and Po, 1976; Feldman, et al, 1982; Skrowronski and Feldman, 1989). An estrogenic substance in the yeast *S. cerevisiae* has been identified as 17β-estradiol (1,3,5(10)-estriene-3,17beta-diol) and this organism also produces an estradiol-binding protein (Feldman et al, 1984). The actual interaction of this hormone and its binding protein has not been shown nor has the purpose that these molecules serve in the yeast been defined.

Mammalian hormones have diverse biological activities in microorganisms (Louria et al, 1960). Insulin inhibited the growth of *Pseudomonas pseudomallei* which causes the disease meliodosis in many type I diabetics (Woods et al, 1993).

17β-estradiol stimulated growth in *Pseudomonas (Burkholderia) cepacia* (Havlichek et al, 1993).

Fungi Respond to Mammalian Hormones

Host hormonal factors have long been suspected of influencing the incidence of some mycoses because of the different rates of disease between men and women (Borelli, 1970). Indeed, hormones have been shown to modulate the pathogenicity of fungi. *Paracoccidioides brasiliensis* infects males and females equally but produces a 48-fold increase in disease among males (Restrepo et al, 1984). In vitro, estradiol inhibits the conversion of the non-pathogenic mycelial form of the yeast *Paracoccidiodes brasiliensis* to the pathogenic yeast form in a dose-dependent manner, with significant inhibition noted at concentrations of 17β-estradiol as low as $1x10^{-10}$ (Loose and Feldman, 1982; Restrepo et al, 1984; Salazar et al, 1988). The difference in the rate of disease may be mediated by this organism's estrogen-binding protein (Loose et al, 1983a; Stover et al, 1986).

A study of the effects of androgens on the growth of *Pityrosporum ovale (Malassezia furfur*) and *C. albicans* showed that most strains of *P. ovale* were inhibited by androgens but that *C. albicans* was not affected (Brasch,1993).

The parasitic phase of *Coccidiodes immitis* is stimulated by 17β-estradiol, progesterone and testosterone (Drutz et al, 1981). Enhancement of the rate of growth of the parasitic phase from arthroconidia was detectable at 17β-estradiol concentrations as low as 10^{-14}M. A dose-related response extended through the physiological range for the normal non-pregnant female ($7.4x10^{-13}$ to $2.4x10^{-10}$) into

the range of unconjugated estradiol found in late pregnancy ($3.7x10^{-7}$ to $8.8x10^{-7}$) (Drutz et al, 1981). The effect of progesterone and testosterone was seen at a concentration of 10^{-6}M.

Hormonal factors are also thought to influence the development of vaginal candidiasis, since these infections are common in pregnancy and while taking oral contraceptives which include high-doses of estrogen (Odds, 1988a; Mitchell, 1984). Vaginal candidiasis has been recognized as one of the most common infectious diseases of pregnancy (Hurley, 1975). Pregnant women have a higher rate of vaginal carriage of candida (Odds, 1988a), and when pregnant and nonpregnant women were experimentally inoculated with *Candida albicans*, a greater incidence of infection occurred in the pregnant women (Bland, 1937). Recurrent candidiasis appears to become clinically evident more often during the premenstrual phase when progesterone is at its highest level and estrogen is somewhat elevated (Odds, 1988a; Hacker and Moore, 1992; Pernoll and Benson, 1987). Experimentally, oophorectomized rats are only susceptible to candidiasis when treated with estrogen (Larsen and Galask, 1984; Sobel, 1992).

C. albicans is dimorphic and some research suggests that the mycelial form contributes more to pathogenesis than the yeast phase (Sobel et al, 1984; Szaniszlo, 1983). Vaginal candidiasis in humans has an increased incidence during periods of higher estrogen levels, such as pregnancy and the premenstrual phase. This disease is rare before puberty or after menopause (Holmberg and Landanska, 1977; Hurley, 1977; Odds, 1988a).

Since progesterone levels also change during these phases of life, researchers have looked at the effect of estrogens and progesterones on *C. albicans* growth and germ tube production. Kinsman (1988) demonstrated an increased percentage of yeast cells producing germ tubes in the presence of estriol, pregnanediol and pregnanetriol. However, germination was stimulated to a lesser extent by 17β-estradiol and progesterone. It was suggested that the capacity of steroids to promote germination of yeast cells was directly proportional to the degree of hydroxylation of these compounds (Kinsman, 1988). Others have reported that 17β-estradiol stimulated germ-tube formation, protein synthesis, and phospholipase activity (Frey et al, 1988). Evidence has also been presented that the hormones progesterone, corticosterone and dexamethasone had no effect on the growth of *C. albicans*, its phase conversion, or glucose oxidation (Loose et al, 1983b).

The complexity of effects increases when one looks at other hormones. Insulin was shown to promote germination of *C. albicans* yeast cells (Nolting et al, 1982). Human luteinizing hormone (hLH) has a mixed record, promoting germination of yeast cells from a late exponential-phase culture (Williams et al, 1990) and inhibiting germination of yeast cells from a stationary phase culture (Paveto et al, 1991). However, the biochemical response of adenylate cyclase in these two studies was similar, implying that more than a G-protein/adenylate cyclase mediated response is involved in the germination response to hLH.

Inhibition and stimulation of fungal growth by steroid hormones at concentrations in excess of 10^{-6}M has been well documented (Chattaway et al,

1963; Buetow and Levedahl, 1964; Moursi and el-Fiky, 1967; Capek and Simek, 1971; Mohr et al, 1973, Stevens, 1989). More recently, steroid hormones at concentrations 10^{-14} to 10^{-6} M have been shown to have an effect on dimorphic fungi such as *Candida albicans* (Kinsman, 1986, 1988; Frey et al, ,1988). Estriol, pregnanediol, and pregnanetriol, in concentrations from 10^{-9} to 10^{-6}M, have been shown to increase the percentage of germination when blastospores of *Candida albicans* were added to serum (Kinsman, 1988) and this mycelial phase is thought to contribute to the disease process. Other steriods (estrone, 17β-estradiol, progesterone) failed to stimulate germination or only stimulated to an insignificant degree (Kinsman, 1988). Another research group reported that estradiol, in nanomolar to millimolar concentrations, stimulated germ-tube formation (Frey et al, 1988). Growth stimulation of certain strains of *C. albicans* was noted at a concentration of 10^{-6}M of 17β-estradiol (Larsen, unpublished results). Other hormones, such as progesterone, corticosterone and dexamethasone, had no effect on growth (at 10^{-6} to 10^{-10} M), phase conversion (at 10^{-6}M) or glucose oxidation (at 10^{-6}M) (Loose et al, 1983a).

Fungal Hormone-Binding Proteins

Fungi appear to interact with mammalian hormones in a manner closely analogous to the interaction of mammalian cells with these compounds. Specific binding and biochemical growth responses to mammalian hormones have been reported in *Candida albicans* and several other fungi (Loose et al, 1981; Loose and Feldman, 1982; Powell et al, 1983a,b; Tanaka et al, 1989; Loose et al, 1983a,b;

Restrepo et al, 1984; Powell et al, 1983a,b; Feldman et al, 1982; Burshell et al, 1984). A remarkable degree of conservation of messenger ligands and receptors is suggested.

17β-estradiol-binding proteins have been found in *Coccidioides immitis* (Powell et al, 1983a), *Candida albicans* (Skrowronski and Feldman, 1989), *Saccharomyces cerevisiae* (Burshell et al, 1984; Feldman et al, 1982), and *Paracoccidioides brasiliensis* (Loose et al, 1983a; Stover et al, 1986). A corticosteroid-binding protein showing high affinity for both corticosterone and progesterone has been found in *Candida albicans* (Loose et al, 1981, 1983b; Loose and Feldman, 1982; Stover et al, 1983; Powell et al, 1983b). Progesterone binding proteins have been identified in the cytosol of *Coccidiodes immitis* (Metzger et al, 1983a) and various dermatophytes (Clemons et al, 1988; Clemons et al, 1989; Schar et al, 1986). Electron immunocytochemistry has localized the estrogen-binding protein (EBP) to the internal tonoplast of the yeast vacuole (Zhao et al, 1995). The fungal vacuole, which is analogous to the mammalian lysosome, is responsible for macromolecular degradation and proteolysis, as well as the maintenance of cytosolic ion and pH homeostasis and metabolite storage (Klionsky, 1990).

Even the hormones human chorionic gonadotropin (hCG) and human luteinizing hormone (hLH) bind specifically to subcellular fractions of *C. albicans*, although the cytosolic fraction bound is different from the mammalian receptor system (Bramley et al, 1990, Bramley et al, 1991). There is also evidence that endogenous hLH-hCG-β-core-like molecules may play a role in regulation of

morphogenesis in *C. albicans* (Bramley et al,, 1991). It is evident that many fungi bind mammalian hormones with high affinity, selectivity, and stereospecificity (Loose et al, 1983; Loose and Feldman, 1982; Powell et al, 1984; Othman, 1988, Skrowronski and Feldman, 1989; Das and Datta, 1985). However, alterations in growth medium or growth phase of the organism may have effects on the expression of binding proteins (Skrowronski and Feldman, 1989). These fungal binding proteins have binding properties distinct from their mammalian counterparts (Powell et al, 1984; Othman, 1988; Skrowronski and Feldman, 1989). For example, the amino acid sequence of the estrogen-binding-protein of *C. albicans* has more homology to oxidoreductase proteins than to the mammalian estrogen receptor (ER) (Madani et al, 1994). The cytosol of several fungal species contains factors which compete with radiolabelled hormone for specific binding sites. These ligands may function as endogenous fungal hormones, but there is no clear evidence of their role yet (Bramley et al,, 1991; Feldman et al, 1984; Miller, 1986; Powell et al, 1983a,b, 1984; Loose et al, 1981). This does raise the question of what function these hormone-binding proteins have in fungi and through what mechanism(s) do they exert their effects.

MECHANISMS OF ESTROGEN

Estrogen has many actions in higher eucaryotes: as a growth factor for follicular cells and oocytes, as a growth factor for human mammary tumor cells and normal mammary cells, and as a transcriptional activator or suppressor of specific genes

of female accessory sex organs (Engel et al, 1978; Huang and Cho-Chung, 1982, 1984).

Growth Factor

Evidence in mammalian and yeast systems indicates that estrogen affects growth through cAMP and adenylate cyclase levels. In oocytes, estrogen maintains high intracellular cAMP preventing the progression to meiosis (Dekel and Beers, 1978). Estrogen increases adenylate cyclase mRNA levels in *S. cerevisiae* cells, delaying meiosis (Hasegawa et al. 1991).

Environmental factors such as hormones or nutrient levels affect the cell cycle of *S. cerevisiae*. When *S. cerevisiae* cells are starved of glucose, they arrest in the G1 phase of the cell cycle. When glucose is added, adenylate cyclase activity is stimulated and subsequently levels of intracellular cAMP rise to the medium. cAMP-dependent protein kinases are then activated, resulting in signals for the cells to grow and divide (Engelberg et al, 1989). Environmental signals to the adenylate cyclase cascade in *S. cerevisiae* are known to be transmitted by guanine-nucleotide-binding proteins (G-proteins) (Matsumoto et al, 1988; Kataoka et al, 1984).

Estrogen can also act as a growth factor for *S. cerevisiae* cells in the early G1 phase through the activation of the adenylate cyclase gene (Tanaka et al, 1989). The constitutive expression of the RAS1 and RAS2 genes of the yeast were not affected, suggesting that some other pathway is being used (Tanaka et al, 1989). The cell cycle, specifically meiosis, in *Saccharomyces cerevisiae* is delayed by the

addition of 17β-estradiol to sporulation-promoting plates. Since intracellular cAMP and adenylate cyclase mRNA levels are increased under these conditions, it is suggested that this delay is mediated by control of the level of cAMP (Hasegawa et al, 1991). The elevated cAMP level may not be due to a direct effect of estrogen, rather to an interaction of estrogen with modulators like IRA1 and IRA2, yeast proteins with homology to mammalian GAP (*ras* GTPase activating protein) (Tanaka et al, 1990).

A receptor for this type of pathway was deduced by researchers working with *Candida albicans* and hLH when adenylate cyclase activity was elevated after incubation of *C. albicans* microsomes with hLH (Williams et al, 1990). Researchers demonstrated that the addition of glucagon to *C. albicans* cells, under conditions conducive to germination, blocked hyphal development (Paveto et al, 1990). They also showed that the Mg+2-dependent, GTP-stimulated adenylyl cyclase was activated by glucagon, hLH, and hCG in a dose-dependent fashion (Paveto et al, 1990, 1991). Further evidence of G-proteins in *C. albicans* comes from investigators who isolated the gene, CAG1, from *C. albicans* using the G-protein α-subunit clone SCG1 of *S. cerevisiae* as a probe (Sadhu et al, 1992). A $G_{\alpha s}$-like protein of 40kDa has been described in *C. albicans* that responds to ADP-ribosylation and is responsible for toxin-induced changes in adenylyl cyclase activity (Paveto et al, 1992). This protein was characterized chemically, using cholera toxin (CTX) and pertussis toxin (PTX), and immunologically, using an antibody raised against the vertebrate G-protein α-subunit (Paveto et al, 1992).

This antibody localized detected proteins in the membranes of *C. albicans* but not the cytosol, consistent with mammalian G-proteins involved in signal transduction from membrane bound receptors (Iyengar and Birnbaumer, 1990; Kaziro et al, 1991; Paveto et al, 1992). No sequence analysis has compared the putative product of the cloned gene with this immunologically detected and functionally implicated protein. It should be noted that despite the close phylogenetic relationships between the two yeast species, *S. cerevisiae* adenylyl cyclase activation has been coupled to monomeric RAS gene products (Toda et al, 1985; Kurjan, 1990) and not to the heterotrimeric G-protein ($G_{\alpha s}$) that has been found in *Candida albicans*. (Matsumoto, 1988; Paveto et al, 1992). Other results indicate the presence of *ras*-like monomeric G-proteins in the cytosol of *Candida albicans,* most likely involved in vesicular transport and secretion (Nagata et al, 1992).

Homologues for the highly conserved CDC28 and G1 cyclins have been cloned from *C. albicans* and suggest that cell cycle regulation may be similar in *S. cerevisiae* and *C. albicans* (Sherlock et al, 1994).

Transactivator

This G-protein mediated system is in apparent contrast to the 'classic' steroid hormone system in mammalian cells. In the mammalian system, the estrogenic hormone is thought to diffuse through the cellular membrane into the cytosol, eventually binding to the nuclear estrogen receptor (ER) and activating the receptor. This receptor then forms a dimer and interacts with the upstream sequences of regulated genes, known as hormone or estrogen response element

(ERE)(Evans, 1988; Green and Chambon, 1988). The ERE usually possesses a dyad axis of symmetry and functions as a *cis*-acting element, having enhancer-like activity (Klein-Hitpass et al, 1988; Cordingley et al, 1987).

Some research has suggested that steroid binding does not affect the receptor's DNA-binding affinity, but biologically active hormone must bind for activation of the receptor-DNA complex (Curtis and Korach, 1990). Protein-protein interactions may occur among homologous receptor complexes, heterologous receptor complexes or receptor-promotor/TATA complexes. These interactions are thought to stabilize transcription factors at the promotors of target genes, inducing a high degree of initiation of transcription (O'Malley, 1990; O'Malley and Tsai, 1992; Klein-Hitpass et al, 1990; Ptashne, 1988; Schule et al, 1988; Mengelsdorf et al, 1995).

The ER protein belongs to a family of proteins which possesses similar structural domains (Evans, 1988; Carson-Jurica et al, 1990). The general structure is composed of a ligand binding domain at the carboxyl terminal (transactivation function 2, or TAF-2, in the ER), a DNA binding domain, and a variable domain at the amino terminal (transactivation function 1, or TAF-1, in the ER) (Kumar et al, 1987; Beato, 1989; Evans, 1988; Carson-Jurica et al, 1990; Pham et al, 1992). TAF-1 and TAF-2 are nonacidic and able to activate transcription independently when separated or synergistically when brought into close proximity (Kumar, 1987; Tora et al, 1989). The activities of both activation domains appear to be cell type and promoter context dependent (Tora et al, 1989). Antiestrogenic compounds do not inhibit the association of the receptor with DNA but rather appear to hinder the

interactions of the receptor with the transcriptional machinery (Pham et al, 1991b). It has been suggested that when antiestrogens function as partial agonists it is by interfering with TAF-2, the ligand binding domain, while allowing TAF-1, the constitutive N-terminal domain, to function (Berry et al, 1990).

Most inactive steroid receptors are sequestered in a complex with the 90-kilodalton (kDa) heat shock protein, hsp90 (Pratt et al, 1992a; Pratt, 1992b; Pratt, 1993; Howard, 1988; Baulieu, 1987; Flower, 1988). Hsp90 is a highly conserved, ubiquitous, and abundant protein that is associated with steroid receptors in cytosol extracts. As a stress protein, hsp90 may assist in protein folding or assembly (Wiech et al, 1992; Hartl et al, 1992; Gething, 1992). The existence of a single HSP90 allele appears to be essential, since repeated attempts to construct and isolate a hsp90/ hsp90 null mutant have been unsuccessful, though generation of a heterozygous hsp90/HSP90 strain was accomplished (Swoboda et al, 1995). The role of hsp90 with receptors may include masking functional domains to maintain an inactive receptor and facilitating hormone-binding by the receptor (Breswick, 1989; Wiech et al, 1992). Hsp90 may participate in the nuclear transport of receptors (Dingwall and Laskey, 1991; Picard et al, 1990b, Guiochon-Mantel et al, 1991) or mediate an aspect of receptor activation, such as phosphorylation.

Phosphorylation plays a role in activation probably through tyrosine kinases (Csermely and Kahn, 1991; Nygard et al, 1991; Nadeau et al, 1992; Auricchio, 1987; Auricchio et al, 1988; Pietra et al, 1995). The Ras-mediated pathway of mitogen-activated protein kinase (MAPK) has been shown to enhance transcription

through phosphorylation of the Ser^{118} residue in the TAF-1 region of the ER (Kato et al, 1995). The activated receptor then binds to the estrogen responsive element of the DNA, forming the base for the initiation complex, and transcription of estrogen responsive genes proceeds with subsequent protein production (Smith et al, 1993; Beato, 1989).

Other proteins and heat-shock proteins such as hsp70, p60, p59 (hsp56), p54, p50 and p23 bind transiently to the receptor complex depending on the cell and receptor type (Onate et al, 1991; Smith et al, 1993b; Tai et al, 1986; Sanchez et al, 1987, 1990). Hormone binding and subsequent disaggregation of the receptors from the heat shock and other proteins promotes activation (transformation) and dimerization of the receptor (Veldscholte et al, 1992).

There is also research that supports a model where hsp90 is lost following ligand binding and the resulting activated ER complex stabilizes the binding of two additional proteins, p48 and p45, when hER binds to ERE. In this model the proportion of liganded hER that does bind ERE, retains hsp70 (Landel et al, 1994). The interaction of ER with components of the transcription initiation complex (specifically TFIIB) is not included in this explanation.

An alternative model proposed recently suggests that the active estrogen receptor (ER) is a monomer or heterodimer and the regulatory role of estrogen on the ER is the induction of protein-protein, but not protein-DNA interactions (Furlow et al, 1993). Other research has shown that some of the classic steroid receptors can be regulated by a membrane receptor agonist (dopamine) in the absence of

ligand. This would demonstrate cross-talk and convergence between major regulatory pathways (Power et al, 1991; Ignar-Trowbridge et al, 1992; O'Malley and Tsai, 1992; Pietra et al, 1995).

Changes in chromatin structure have been implicated in the mechanism of activation of several steroid-responsive promoters (Nardulli and Shapiro, 1992; Pham et al, 1991a; Ishibe et al, 1991; Reik et al, 1991; Carr and Richard-Foy, 1990; Richard-Foy and Hager, 1987; Hecht et al, 1988). There appears to be a strong correlation between chromatin structure disruption at promoter regions and the ability of promoter-bound ER derivatives to transactivate (Pham et al, 1991a). Research has also indicated that transactivation regions of the ER also play a role in mediating local chromatin structure disruption (Pham et al, 1991b; Pham et al, 1992).

The metabolic breakdown products of steroids have recently become the focus of research. In trying to understand the good effects of estrogen on bone and its bad effects on breast and uterine cancer, researchers discovered that the gene encoding transforming growth factor-β3 (TGF-β3), that estrogen activates in living animals, is not activated in cultured bone cells. An intermediate, 17-epiestriol, formed as the body degrades estrogen for excretion, is the ligand necessary for the activation of this gene through a DNA binding mechanism that is different from the ERE (Yang et al, 1996). This may be the first of many alternative paths that estrogen takes to affect different tissues. Similar alternatives may exist for its effects on *Candida*.

Fungi produce molecular messengers which act as hormones, regulating sexual development and cross-reacting between species. Some of these molecules also cross-react with mammalian systems. Mammalian hormones interact with fungi, affecting fungal growth, germination, and pathogenesis of disease. These hormones may exert their influence through the hormone-binding proteins that have been found in fungi. These proteins may also participate in fungal pathways that respond to endogenous fungal 'hormones'.

Estrogen acts as a growth factor in mammalian and fungal cells through a pathway that uses G-proteins, cAMP, and adenylate cyclase levels. Estrogen's role as a transcriptional activator in mammalian cells uses a pathway that includes a hormone receptor, receptor associated proteins, DNA binding and/or protein-protein interactions.

Evidence for the 'Classical Pathway' in Yeast

The basic transcription machinery is remarkably conserved between mammals and yeast. The sequence of the large subunit of RNA polymerase II from human cells and yeast show considerable homology (Allison, el al, 1988). The TATA box binding protein, TFIID, is functionally interchangeable and shows a high degree of structural homology (Cavallini et al, 1988). The mechanisms of transitional activation appear to be conserved between mammals and yeast also. The yeast activator GAL4 functions in mammalian systems (Webster et al, 1988) and mammalian steroid hormone receptors activate transcription in yeast (White et al, 1988; Schena and Yamamoto, 1988; Mak et al, 1989; Wright et al, 1990a).

The yeasts *Saccharomyces cerevisiae* and *Candida albicans* have been transformed with the gene for the human estrogen receptor (hER) and the estrogen responsive element (ERE) (White et al, 1988; Metzger et al, 1988). Reporter gene expression indicates that the activity of hER in yeast can be regulated by ligands in a fashion very similar to that in mammalian cells, that both TAF-1 and TAF-2 function in yeast (Pham et al, 1991a; Metzger et al, 1992), and that these yeasts have the accessory proteins necessary for the classic system (Metzger et al, 1988; McDonnell et al,1991; Lyttle et al, 1992). The strict mammalian hormone-receptor-transactivation mechanism may not be what is at work in *C. albicans*, however.

Synergism and Cross Talk

There may also be multifactor regulation of pathways and genes in the microbe. Synergistic and permissive effects of two or more hormones (or hormone metabolites) upon the expression of a single gene have been documented in mammalian systems (Evans et al, 1982; Horwitz, 1978). Estradiol induction of ovalbumin transcription in oviduct tissue occurs only in the presence of insulin, while neither hormone alone is active (Evans et al, 1981). Sometimes the permissive effects reflect the induction by one hormone of functional receptors for the second: estradiol induces the appearance of progesterone receptors and androgen receptors in oviduct cells, which then bind their respective ligands and induce ovalbumin synthesis (Tokarz, 1979). In rat hepatoma (HTC) cells dexamethasone permits cAMP to act as a strong inducer of the tyrosine aminotransferase gene, where cAMP fails to stimulate expression by itself

(Granner, 1976). In osteoblast cells estradiol increases the receptor number for vitamin D3 (Ishibe et al, 1995). Whether these phenomena are the result of the differential activities of two or more distinct enhancers upon a single promoter or are due to postranscriptional and/or posttranslational strategies have yet to be discovered.

One method for synergy has been elucidated for estrogenic environmental chemicals, such as toxaphene and chlordane (Simons, 1996). When studied singly in a yeast system transfected with the human estrogen receptor (ER) and a lacZ reporter plasmid, these chemicals showed low potencies, suggesting they would have little biological effect. However, when the two weakly estrogenic compounds were tested, the combination was 1000 times as potent (Arnold et al, 1996). Ovarian and phytoestrogens also act synergistically in this yeast system (Miksicek, 1994; Arnold et al, 1996). The researchers also demonstrated the synergistic effect in human endometrial cells, avoiding the criticism that the yeast expression system for mammalian steroid transactivation is not entirely comparable to expression in mammalian cells (Wrenn and Katzenellenbogen, 1993; Lyttle et al, 1992).

Synergism is not unique to environmental chemicals or cross-species interactions. In mammalian cells, signaling pathways involving protein kinases activated by growth factors play a role in estrogen action (Ignar-Trowbridge et al, 1992, 1993; Korach, 1992). It has been shown that phosphorylation of the human estrogen receptor (hER), which is required for full activity, can be accomplished

through the Ras-MAPK (Mitogen-activated protein kinase) cascade of the growth factor signaling pathways (Kato et al, 1995; Peitras, 1995) and through the action of casein kinase II (Arnold et al, 1995).

Other research has shown that selected steroid hormone receptors can be activated in vitro in a ligand-independent manner by a membrane receptor agonist, the neurotransmitter dopamine (Power et al, 1991). Further research into this phenomenon indicates that ligand and ligand-independent activation of the ER initiate from distinct pathways and that the latter may occur in a variety of target tissues subject to modulation by receptor ligands (Smith et al, 1993). Dopamine is regarded as involved in the regulation of postsynaptic gene expression in the central nervous system (Rogue and Malviya, 1994; Chio et al, 1994). Dopamine receptors use various second messenger systems, such as one that requires protein kinase C but not cAMP and one that uses G_i-proteins (Di et al, 1993). The evidence for interaction between the nervous system and hormonal regulation has implications for physiological research but also for the idea of cross-talk between signaling pathways.

The Jak-STAT signal transduction pathways, which consists of protein tyrosine kinases and tyrosine-containing signal transducers, has been shown to be triggered by interferon (α and γ) and other cytokines as well as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and prolactin (Darnell et al, 1994; Argetsinger et al, 1993; Cosman, 1993; Dusanter-Fourt et al, 1994; Ihle et al, 1994a, 1994b; Zhong et al, 1994a,b; Campbell et al, 1994). The common theme

of this pathway seems to be polypeptide ligands (Ihle, 1994c). Although a common pathway may be used by these different messengers, the specificity of gene transactivation is probably designated by the receptor. It is intriguing that EGF has been demonstrated to activate both the G-protein/adenylate cyclase system and the Jak-STAT mechanism. The EGF receptor is membrane bound and the Jaks were originally considered to be only soluble kinases.

Enhanced transcriptional activity of the ER has been observed in the presence of some antiestrogens (ICI 164,384, tamoxifen) and elevated intracellular cAMP (Ince et al, 1994). It may be that other agents that increase the intracellular cAMP level will enhance the activity of the ER.

More and more evidence is accumulating for an elaborate coupling of the steroid response pathway with other cellular signaling transduction mechanisms in mammalian cells. Treatment with epidermal growth factor (EGF) and imposition of cell stress by heat shock or inhibition of protein synthesis enhanced glucocorticoid response in a human breast cell line containing a hormone-responsive luciferase reporter gene (Nordeen et al, 1994). Protein kinase homologues in *S. cerevisiae* (HOG1) and mammalian (p38) cells are both tyrosine phosphorylated after extracellular changes in osmolarity, indicating a similar signaling pathway in yeast and mammals (Han et al, 1994). The more we investigate, the more similarities in regulation we demonstrate between mammalian cells and other eukaryotic cells like *Saccharomyces* and *Candida*.

The basic transcriptional machinery is remarkably conserved between mammals

and yeast as demonstrated by the homology between the large subunit of RNA polymerase II from human cells and yeast (Miksicek et al, 1986; Miksicek, 1994; Allison, 1988). The yeast and human TATA box binding proteins, TFIID, are functionally interchangeable and show a high degree of structural homology (Cavallini, 1988). The yeast activator GAL4 functions in mammalian systems, indicating that the mechanisms of transcriptional activations are conserved between mammals and yeast (Webster et al, 1989). A highly conserved region in the hormone-binding domain of the hER functions as an efficient transactivation domain in yeast (Pierrat et al, 1994). Mammalian glucocorticoid receptor derivatives enhance transcription in yeast (Schena and Yamamoto, 1988). Mammalian steroid hormone receptors activate transcription in yeast (White et al, 1988; Mak et al, 1989; Wright et al, 1990a; Lyttle et al, 1992; Metzger et al, 1988). On the other hand, the yeast reporter-expression transfection system, using the human estrogen receptor (hER) on one plasmid and the vitellogenin estrogen responsive element (ERE) and reporter gene on another plasmid, do not entirely mimic the mammalian system. For instance, antiestrogens can have agonist activity in this yeast system (Wrenn and Katzenellenbogen, 1993) and different regions of the hER have independent transactivation activity in yeast and mammalian cells (Metzger et al, 1992, 1995).

MATERIALS AND METHODS

STRAINS

The strains used were all clinical isolates from the culture collection of Dr. Bryan Larsen, Marshall University. Genus and species were confirmed by growth on a selective and differential medium (Biggy agar) and germ tube production (GTI medium). Inoculum suspensions used in the experiments, unless otherwise stated, were approximately $1X10^5$ CFU/ml as determined by hemacytometer count and confirmed by counting serial dilutions of organisms as colony forming units (CFU). The six strains used in the germ tube and extracellular protein experiments were randomly chosen from the culture collection. The six strains used in the heat shock protection and acid proteinase experiments were selected based on their response to estrogen in other growth experiments in this laboratory (Gujar, in press). Two strains which showed stimulation (J23, 47), two which were neither inhibited nor stimulated (1021,1441), and two which were inhibited (JMMS31, 8749) by 17β-estradiol (1,3,5(10)-estriene-3,17beta-diol) were chosen. The *Escherichia coli* strain XL-1 Blue was used in the transformation and plasmid amplification and plasmid maintenance.

PLASMIDS AND VECTORS

Plasmid YEPE-10 which contains the human estrogen receptor (ER) on a XhoI/SacI fragment, courtesy of B.W. O'Malley (McDonnell et al,1991), was used as the source for the ER probe. Plasmid 1041 contains the *Candida albicans'*

URA3 gene (which encodes orotidine-5'-monophosphate decarboxylase), was used courtesy of S. Scherer (Goshorn et al, 1992). The 1041 plasmid was used as a template for PCR amplification of the CaURA3 gene which was used as a control probe. These plasmids were maintained in *Escherichia coli* strain XL-1 Blue in LB medium and stored with 10% sterile glycerin in LB media at -70°C. The plasmid vector Bluescript SK+ (Stratagene Inc., La Jolla, Ca.) was used to subclone the putative estrogen receptor (ER) gene from the *C. albicans* genome into *E. coli.*

MEDIA

Biggy agar (Sigma Chemical Co., St. Louis, Mo.) was used for confirmation of *Candida albicans* strains. A chemically defined media (CD) previously reported by Shah (1992), (.001g $MgSO_4$, 14.0 g K_2HPO_4, 6.0 g KH_2PO_4, 5.0g NaCl per liter and 1.25% (w/vol) glucose), was used for the growth studies and extracellular protein production studies. *E. coli* strains were maintained in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2; Sigma Chemical Co., St. Louis, Mo.). Ampicillin (100ug/ml final concentration; Bristol-Myers) and tetracycline (50ug/ml final concentration; Sigma) were added to select for transformants. For acid proteinase induction studies yeast carbon base (YCB, Difco) medium plus 0.2% (wt/vol) bovine serum albumin (BSA, Sigma) was used (YCB+). BSA was added to a final concentration of 0.2% from a 2% filter-sterilized stock solution. The germ tube induction (GTI) medium used was 3.5% BSA (from 10% filter sterilized stock, Sigma) and 3.5% peptone (from 10% filter-sterilized stock solution, Sigma) in phosphate buffered saline (PBS). Yeast Nitrogen Broth with

amino acids (YNB w/aa) and YNB without amino acids (YNB, Difco) were used for maintaining cultures of *C. albicans* and for overnight growth in the extracellular protein production studies. Sabouraurd's broth and plates were used for routine mainenance of *C. albicans* cultures and viable organism counts (colony-forming-units, CFU, counts).

STATISTICAL METHODS

Statistical calculations were done with the Microstats program. Paired observations in the student T test were applied to studies with continuous variables (extracellular protein, acid proteinase, and heat shock survival).

GROWTH STUDIES

C. albicans strain 9495 was grown overnight in 10 ml of YNB at 37°C, with shaking, to a density of 10^7 CFU/ml. The cultures were centrifuged at 2000 rpm for 20 minutes, washed twice with sterile water and resuspended to a cell density of 5 x 10^6/ml. This suspension was added to 50 ml of CD medium in 250 ml flasks for a final cell density of 2 x 10^3/ml. These flasks also contained vehicle only (control) or 10^{-6}M of 17β-estradiol, α-estradiol, testosterone,or cholesterol. The solvent for the hormones was ethanol, which was used in the control cultures at a 1% concentration. Previous studies comparing growth with and without 1% ethanol indicated that 1% ethanol had no detectable effect on the growth of *C. albicans*. These cultures were incubated at 37°C with shaking. Absorbance at 650nm and viable organism counts (CFU/ml) were determined at the time of inoculation (0), 7, 31, 53, and 72 hours. A final biomass on 5 ml of well mixed culture was determined

after 7 days of incubation. Cultures were centrifuged, washed with sterile water and dried down (Speed-Vac, Savant Instruments, Inc., Farmingdale, NY) in weighed tubes.

Viable organism counts were performed in the extracellular protein production and acid proteinase studies by serial dilutions of the well-mixed cultures in sterile water. Aliquots of the dilutions were inoculated onto Sabouraud agar, incubated overnight at 37°C, the colonies counted the next day and the viable organisms calculated for each milliliter.

EXTRACELLULAR PROTEIN PRODUCTION

The six strains (WVU23, 26MZ, Y7410, 1441, 9495, J22) were grown on Sabouraud's agar at 25°C overnight. Suspensions were made in CD medium to avoid osmotic stress, with a final concentration of $1X10^5$ CFU/ml. 100µl of each suspension were inoculated into pairs of 10ml of YNB with dextrose, with and without 17B-estradiol (10^{-6}M), and incubated at 37°C with shaking. Final viable organism counts (CFU/ml) were determined on day four. Aliquots of the cultures were taken at 1,2 and 4 days and centrifuged at 2000 rpm, for 20 minutes. Total protein was determined on the supernatant by the BCA method. Aliquots were also taken at the specific time points from uninoculated medium, centrifuged, and total protein assayed as a negative control. Protein standards of bovine serum albumin (BSA) in distilled, sterile water were used at concentrations of 50, 100, 150, 200, 250 ug/ml.

The enhanced protocol, with 0.1 ml of sample and 60°C incubation, of the BCA Protein Reagent (Pierce) was used to determine total protein. This reagent is based on the bicinchoninic acid method of Smith et al(1985).

GERMINATION STUDIES

Six strains of *Candida albicans* were grown on Sabouraud's agar overnight at 30°C, suspended in 1.0 ml of phosphate buffered saline (PBS) to a concentration of $2X10^5$ CFU/ml. 50 µl of this suspension was added to 500ul of the GTI medium which also contained a hormone or the control vehicle, and incubated at 37°C. A set of eight steroids and a control was inoculated with each strain. The hormones (17β-estradiol, testosterone, progesterone, hydroxycortisone, prednisone), antagonist (tamoxifen), synthetic estrogenic analog (diethylstilbestrol) and hormone precursor (cholesterol) were dissolved in ethanol and the final concentration in the germ tube induction medium was 10^{-6}M. The concentration of ethanol in the GTI was 1%. 5 µl aliquots were taken at 2 and 4 hours and examined microscopically on a hemacytometer for germ tube formation. 200 cells were counted for each sample at each time point.

HEAT SHOCK PROTECTION

5 ml of YCB medium plus 0.2 % BSA was set up in duplicate with and without 17B-estradiol (10^{-6}M). Each strain (J23, 47, 1021, 1441, JMMS31, 8749) was grown overnight on Sabouraud's agar at 37°C. Suspensions were made in YCB for a concentration of $1X10^5$ CFU/ml. Each pair was inoculated with 50 µl of a suspension and incubated overnight at 25°C with shaking. The cultures were

centrifuged at 2000 rpm for 20 minutes, the supernatant discarded, and cells washed with sterile PBS twice. The cells were resuspended in YCB plus BSA to a concentration of about 2-5 x 10^6 CFU/ml and divided for different temperature exposures (22°C, 37°C, and 50°C). Samples were taken at time 0 and 15 minutes to determine the concentration of viable organism (CFU/ml). This experiment was done twice.

ACID PROTEINASE SECRETION

Induction/Growth Medium

C. albicans strains were grown on Sabouraud's agar overnight at 37°C. Cell suspensions at a concentration of approximately 2X10^5 CFU /ml of washed cells were made in YCB+. 50 µl of this suspension was introduced into 3 ml of YCB+ medium, incubated at 30° - 35°C with shaking, for 48 hours. Each strain was inoculated into two tubes of YCB+ medium: one contained 10^{-6}M 17 β-estradiol in ethanol (50 µl of 10^{-4}M 17β-estradiol stock) and the other had only the 50 µl of ethanol solvent added. 10 µl aliquots for viable organism counts were taken before centrifugation.

Assay

1 ml aliquots of YCB culture were centrifuged at 2000 rpm for 10 minutes. 0.3 ml of the supernatant was used for what plus 1.2 ml of the substrate medium (1% BSA in 50mM sodium citrate adjusted to pH 4 with 1N HCl). This was incubated for 1 hour at 37°C. The reaction was stopped with 0.8 ml of 10% trichloracetic acid (TCA), chilled for 1 hour at 4°C, and then centrifuged at 2000 rpm

for 20 minutes at 4°C. The supernatant was measured for absorbance at 280 nm against a water blank. The substrate control was composed of 1.2 ml of substrate medium, 0.3 ml of uninoculated growth medium and 0.8 ml TCA. One unit of enzyme activity was defined as the amount of enzyme causing a change in A_{280} = 0.1 in 30 minutes (Milewski et al, 1994). This method measures the amount of tryptophan released from the protein by the proteinase during the digestion process.

SOUTHERN BLOT ANALYSIS

Southern analyses of restriction endonuclease -digested genomic DNA and plasmids were performed as described in Current Protocols in Molecular Biology (1994). Restriction enzymes which included EcoRI (for genomic DNA digests), HindIII (for digestion of the λ phage for DNA size markers), BamHI, XbaI, XhoI, SacI (used to cut the probes from the plasmids) were obtained from New England Biolabs, Beverly, Mass. All probes were labeled with digoxigenin (DIG High Prime DNA kit, Boehringer Mannheim). Detection was by chemiluminescence (DIG High Prime DNA Labeling and Detection Kit, Boehringer Mannheim). Salmon testes DNA (Sigma) was added to the hybridization solution to eliminate non-specific binding by the probe.

PROBES

The control probe used was a 600 bp BamHI/XbaI fragment from the *Candida albicans* URA3 gene for orotidine-5'-monophosphate decarboxylase amplified from plasmid 1041. The sequence of URA3 was obtained from the

EMBL/GenBank/DDBJ database and was published by Losberger and Ernst, (1989).

The estrogen receptor (ER) probe was cut from the YEPE-10 plasmid, on a XhoI/SacI segment about 1.9kb in size, and purified.

PLASMID, DNA FRAGMENT AND GENOMIC DNA PURIFICATION

Plasmid DNA was purified with the Wizard mini-prep kit (Promega, Madison, WI). The DEAE paper (NA45 from Schleicher & Schuell, Keene, NH) method of purifying DNA fragments from a 1% agarose gel was used following the method in Current Protocols in Molecular Biology (1994) 2.6.4. Genomic DNA was extracted and purified with the Wizard Genomic DNA Purification kit (Promega), using the yeast method with yeast lytic enzyme (ICN Biomedicals, Inc., Irvine, CA).

RESULTS

GROWTH STUDIES

Growth rate was determined on strain 9495 to confirm previous observations in this laboratory that estrogen, in the form of 17β-estradiol (1,3,5(10)-estriene-3,17beta-diol), stimulates overall growth of this *C. albicans* strain. Viable organisms were counted at the time of inoculation, 7, 31, 53 hours, and 3 days. The cultures with estradiol showed a steady, linear increase in organisms that started earlier and was greater than the cultures with other additives (α-estradiol, testosterone, cholesterol and ethanol control (1% final concentration)). Between 53 hours and 3 days the estradiol-treated cultures showed a sharp increase in growth, with a final count at 3.0 CFU x 10^6/ml. Cholesterol-treated cultures also increased during this time, with a final count at 1.0 CFU x 10^6/ml. The other cultures had final counts less than 0.5 CFU x 10^6/ml. This strain demonstrated such dramatic stimulation by estradiol after 53 hours of incubation in a minimal medium but did not show the same response in later studies with other growth media and shorter incubations. In this growth rate experiment the difference between the estradiol-treated culture and the other treatments was apparent after seven hours, although not significant until 53 hours.

When the biomass of the different cultures was measured after 7 days (Figure 2), the culture with 17β-estradiol had significantly higher dried biomass than the control (over 15 times higher). Even compared to the other steroids, 17β-estradiol cultures created 2-4 times more biomass. This indicates that the difference in the

Figure 1

Growth Rate

The effect of different steroidal compounds (10^{-6}M final concentration of steroidal compounds, 1% ethanol final concentration of vehicle) on the growth rate of *C. albicans* strain 9495. Cultures were incubated for 3 days in CD medium at 37°C with shaking. Viable organism counts were performed by making serial dilutions of the well-mixed cultures and inoculating aliquots onto Sabouraud's agar. Colonies formed represent one viable organism. This experiment was performed once.

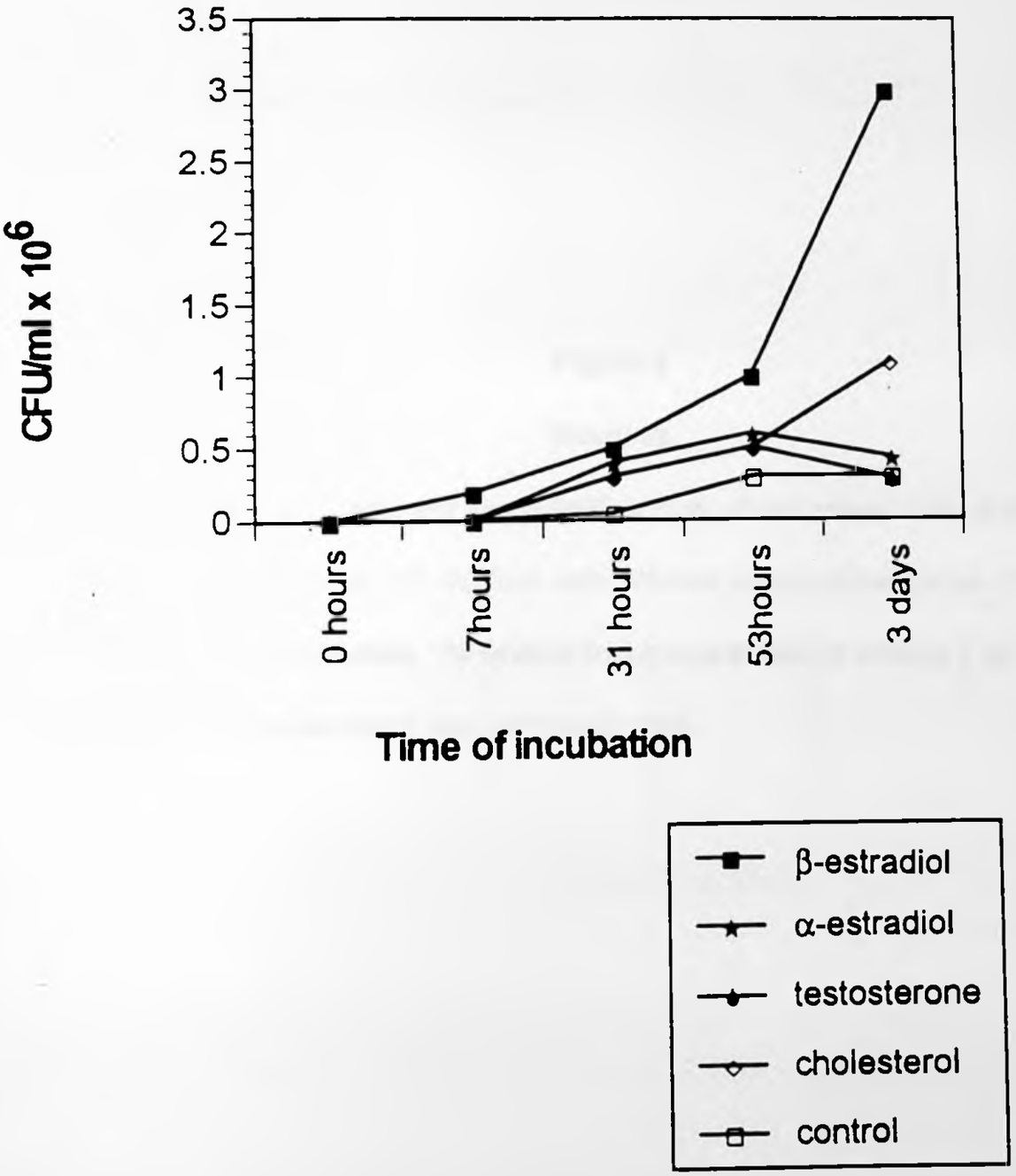
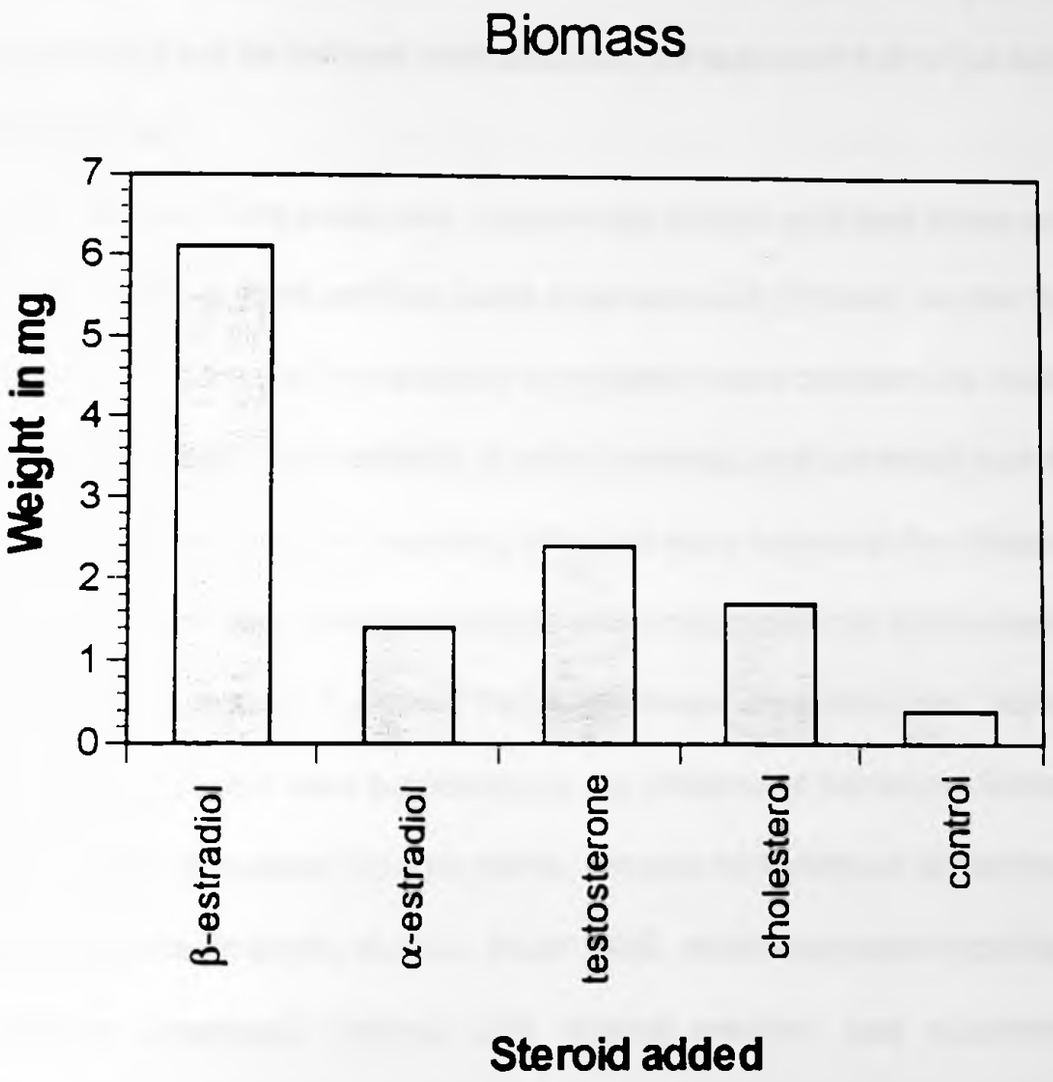

Growth of Candida albicans
CFU/ml x 10^6
3.5
3
2.5
2
1.5
1
0.5
0
0 hours
7hours
31 hours
53hours
3 days
Time of incubation
β-estradiol
α-estradiol
testosterone
cholesterol
control

Figure 2

Biomass

Dried biomass of *C. albicans* strain 9495 in 5 ml of well-mixed culture taken after 7 days of incubation in CD medium with different steroidal hormones (10^{-6}M final concentration of hormones, 1% ethanol final concentration of vehicle) at 37°C with shaking. This experiment was performed once.


Biomass
Weight in mg
7
6
5
4
3
2
1
0
β-estradiol
α-estradiol
testosterone
cholesterol
control
Steroid added

number of organisms produced in the estradiol-treated cultures remains higher than the number of organisms in the other treatments after the three day endpoint of the growth rate study. The non-estradiol-treated cultures may have a growth spurt after three days but the biomass produced does not approach that of the estradiol-treated cultures.

Other studies (acid proteinase, extracellular protein, and heat shock survival) involved measuring initial and final viable organism count (CFU/ml) so that the total values could be corrected for variations in replication rates between the strains and between the treated (with estradiol or other hormone) and untreated (control).

Other work done in this laboratory after this study looked at the effect of 17β-estradiol on colony size when the hormone was incorporated into Sabouraud's agar plates (Gujar, in press). It showed that some strains were stimulated, some were not affected, and some were suppressed by the presence of estradiol. Some of the strains showing stimulation on agar plates, showed no significant difference in the viable organisms (in broth) studies. Strain 9495, which responded significantly to estradiol in chemically defined (CD) minimal medium, was suppressed on Sabouraud's agar incorporating estradiol. Most of the other strains also demonstrated variable effects of estradiol on growth in different culture conditions. Two strains showed a consistent and significant response or a lack of response to estradiol in the growth medium. Strain J23 was stimulated by estradiol in YCB+ medium, after 24 hours at 22°C and 48 hours at 30 - 35°C , and on Sabouraud's agar with estradiol after 24 hours of incubation at 37°C. Strain 1441 showed no

response to estradiol in YNB medium, with four days of incubation at 37°C, in YCB+ medium, with 24 hours of incubation at 22°C and on Sabouraud's agar, with 24 hours of incubation at 37°C.

For most of the strains examined, the effect estradiol had on the growth of the yeast depended upon the growth medium and length of incubation. In one system estradiol might stimulate and in another it might inhibit growth. One strain was consistently stimulated by estradiol and one strain consistently had no response.

EXTRACELLULAR PROTEIN

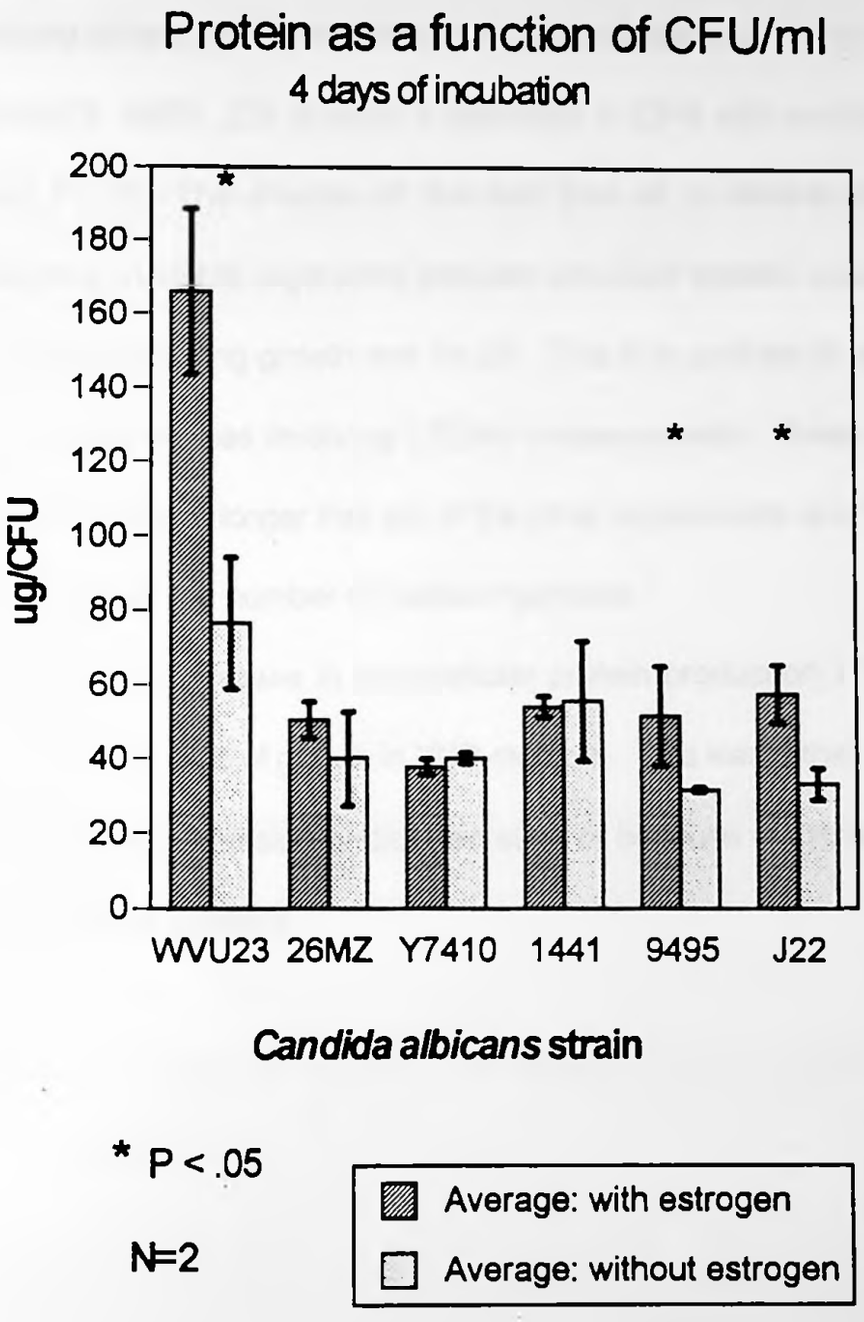
Other researchers have reported that protein production increases under the influence of estradiol (Frey et al, 1988). An increase in extracellular protein can indicate an increase in protein adhesins or secreted enzymes and can be a reflection of generalized metabolic stimulation.

When the protein production per cell was calculated, three strains, WVU23, 9495 and J22, showed a significant difference ($P<.05$), with estradiol increasing extracellular protein (Figure 3). These same strains showed a significant decrease in the number of viable organisms after the four day incubation. These strains may be more susceptible to cell lysis after exposure to estradiol, releasing cytosolic proteins into the growth medium. Analysis of all of the strains showed that the difference in protein production between treatments approached statistical significance with $P=.07$. This experiment was performed twice.

Figure 3

Extracellular Protein Production

Six strains of *C. albicans* were incubated in YNB with dextrose, with and without 17β-estradiol (10^{-6}M), at 37°C with shaking for 4 days. Aliquots of the well-mixed cultures were taken for viable organism counts. Total protein was measured on centrifuged supernatant with bicinchoninic acid method. The amount of protein produced by each viable organism was calculated (μg/CFU). This study was repeated twice.


Protein as a function of CFU/ml
4 days of incubation
ug/CFU
200
180
160
140
120
100
80
60
40
20
0
*
*
*
WVU23
26MZ
Y7410
1441
9495
J22
Candida albicans strain
* P < .05
N=2
Average: with estrogen
Average: without estrogen

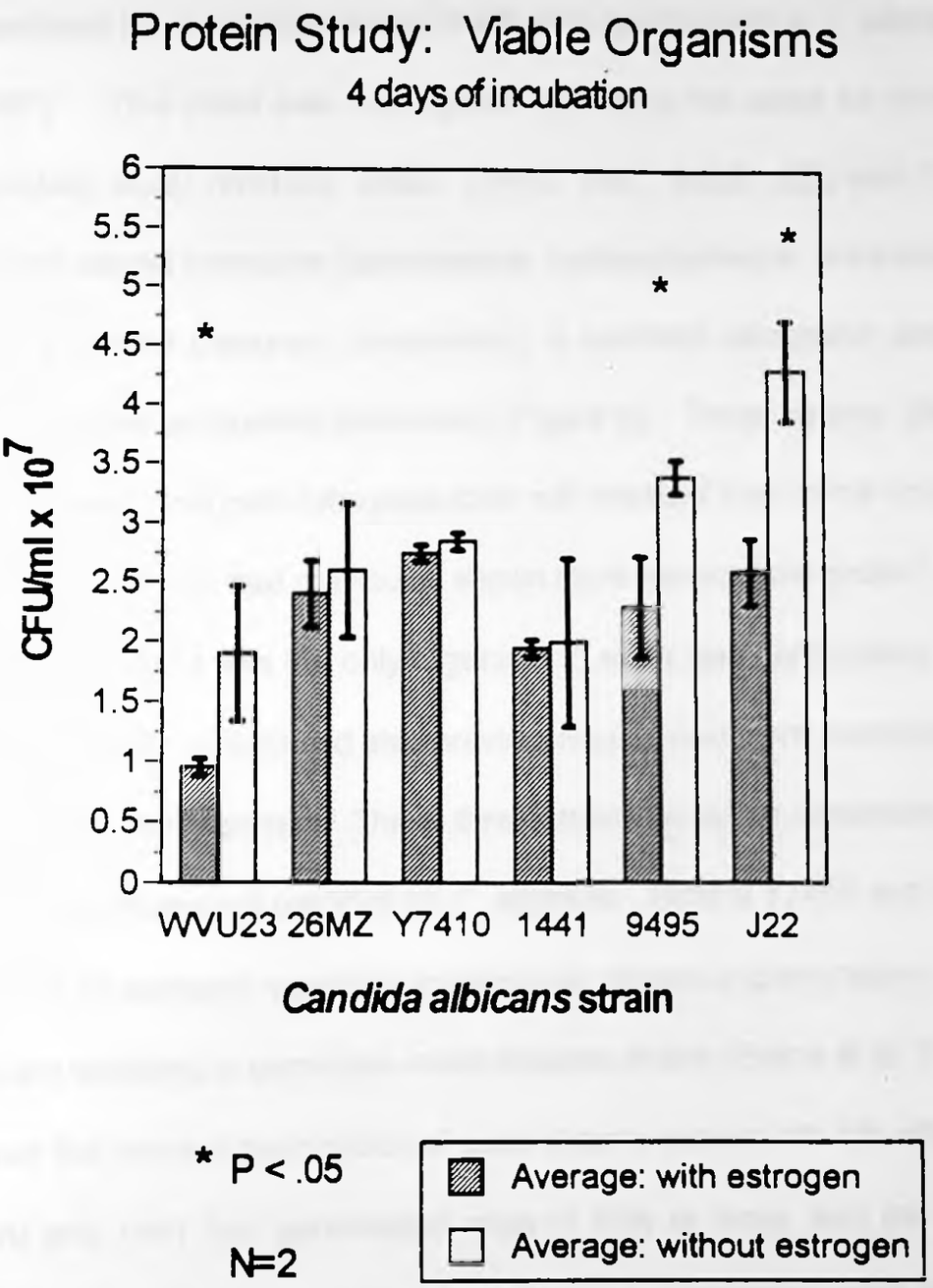
The final CFUx10^7/ml were counted and analyzed by strain and three strains showed a significant difference in growth between estradiol-treated and the controls (Figure 4). WVU23, 9495, J22 showed a decrease in CFU with estradiol and significance was $P<.05$. The analysis of the data from all six strains showed a significant difference in viable organisms between estradiol treated cultures and controls, with estradiol inhibiting growth and $P<.05$. This is in contrast to what was generally found in other studies involving CFU/ml measurements. These cultures were incubated for four days, longer that any of the other experiments and that may have negatively affected the number of viable organisms.

Estradiol induced an increase in extracellular protein production in three *C. albicans* strains after four days of growth in YNB medium. This was either because of an increase in protein synthesis and/or secretion or because of increased cell lysis, releasing cytosolic proteins.

Figure 4

Viable Organisms in Extracellular Protein Study

Colony-forming-units (CFU/ml) were determined by serial dilutions of well mixed cultures of six strains after 4 days of incubation at 37°C with shaking in YNB (with dextrose) and with 17β-estradiol (10^{-6}M) or with ethanol (1% final concentration). This study was repeated twice.


Protein Study: Viable Organisms
4 days of incubation
CFU/ml x 10^7
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
WVU23
26MZ
Y7410
1441
9495
J22
Candida albicans strain
* P < .05
N=2
Average: with estrogen
Average: without estrogen

GERMINATION

Other researchers have reported estradiol affecting germination in *C. albicans* (Kinsman, 1987). This effect was investigated by testing the same six strains used in the protein study (WVU23, 26MZ, Y7410, 1441, 9495, J22) with 17β-estradiol and other steroid hormones (testosterone, hydroxycortisone, prednisone, progesterone), a steroid precursor (cholesterol), a synthetic estrogenic analog (DES) and an estrogen antagonist (tamoxifen) (Figure 5). Three strains, 26MZ, 9495 and J22, showed more germ tube production with estradiol than in the control. Two of these, 9495 and J22, had previously shown more extracellular protein with estradiol. Strain WVU23 was the only organism to show less germination with estradiol than its control. WVU23 had also previously produced more extracellular protein when grown with estradiol. These three strains would be candidates for further study on the influence of estradiol on *C. albicans*. Strains Y7410 and 1441 showed no effect by estradiol regarding extracellular protein or germination.

The inherent tendency to germinate varies between strains (Evans et al, 1975). Figure 6 shows the percent germination of each strain's control with 1% ethanol. Strains Y7410 and 1441 had germination rates of 40% or more, and the other strains had rates less than 20%. A wide range of innate germination rates among these strains is apparent.

Table 1 presents the germ tube production by strain and hormone as a percent of each strain's control, with inhibition by the compound showing germination less than 100 and stimulation showing germination more than 100. Estradiol stimulated

Figure 5

Germ Tube Production

Effect of steroidal hormones on the germination of six *Candida albicans* strains. After overnight growth on Sabouraud's agar at 30°C, washed cells were incubated one hour at 37°C in germ tube induction (GTI) medium with one of eight different compounds or with only the solvent (ethanol control, final concentration was 1%). 200 cells were counted microscopically and the percent germination calculated. The experiment was performed once.

Germ Tube Production

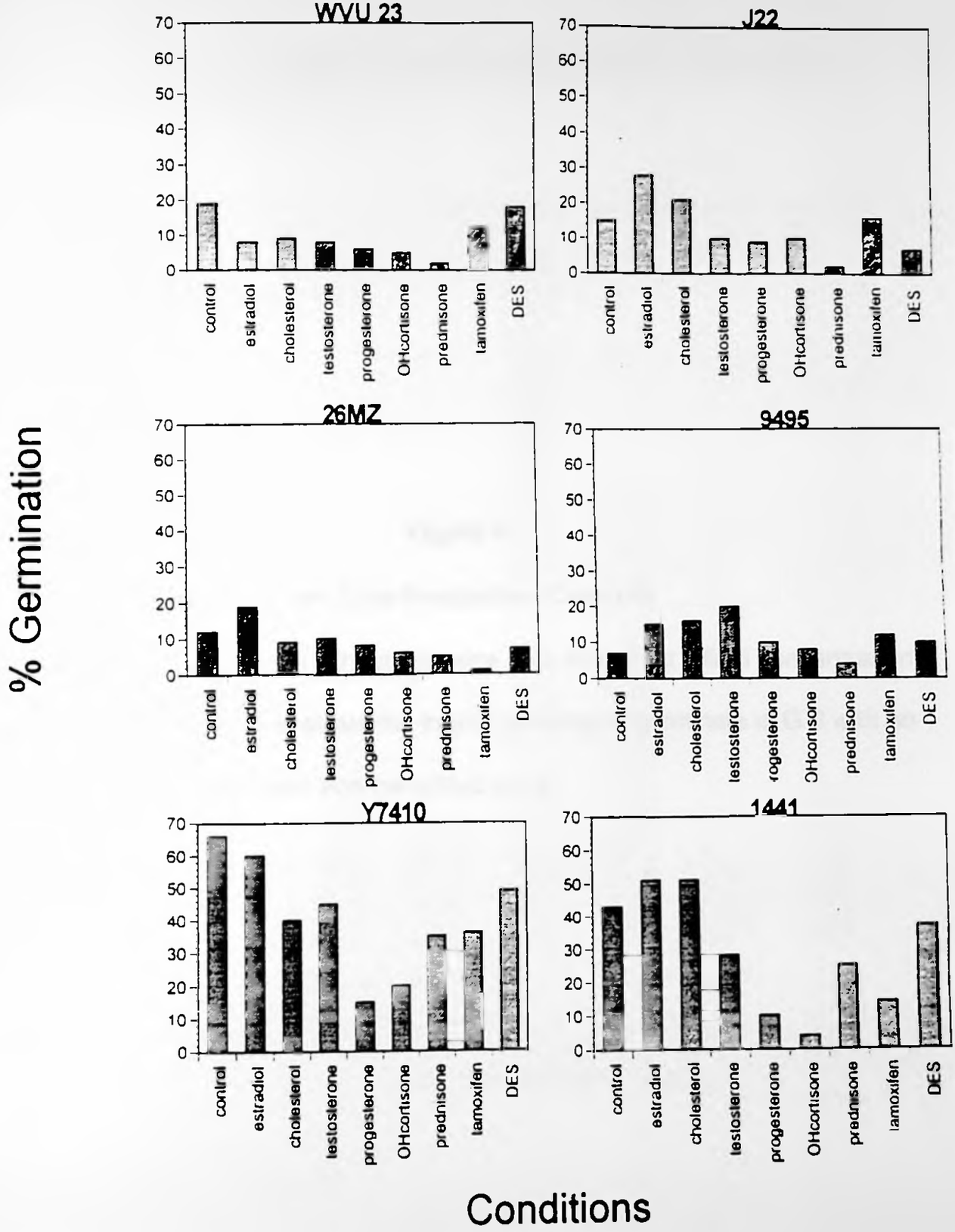


Figure 6

Germ Tube Production: Controls

Comparison of the control (without hormone, with ethanol at a final concentration of 1%) for each strain to evaluate the innate tendency to germinate in GTI with no hormones. The experiment was performed once.

Germ Tube Production: Control

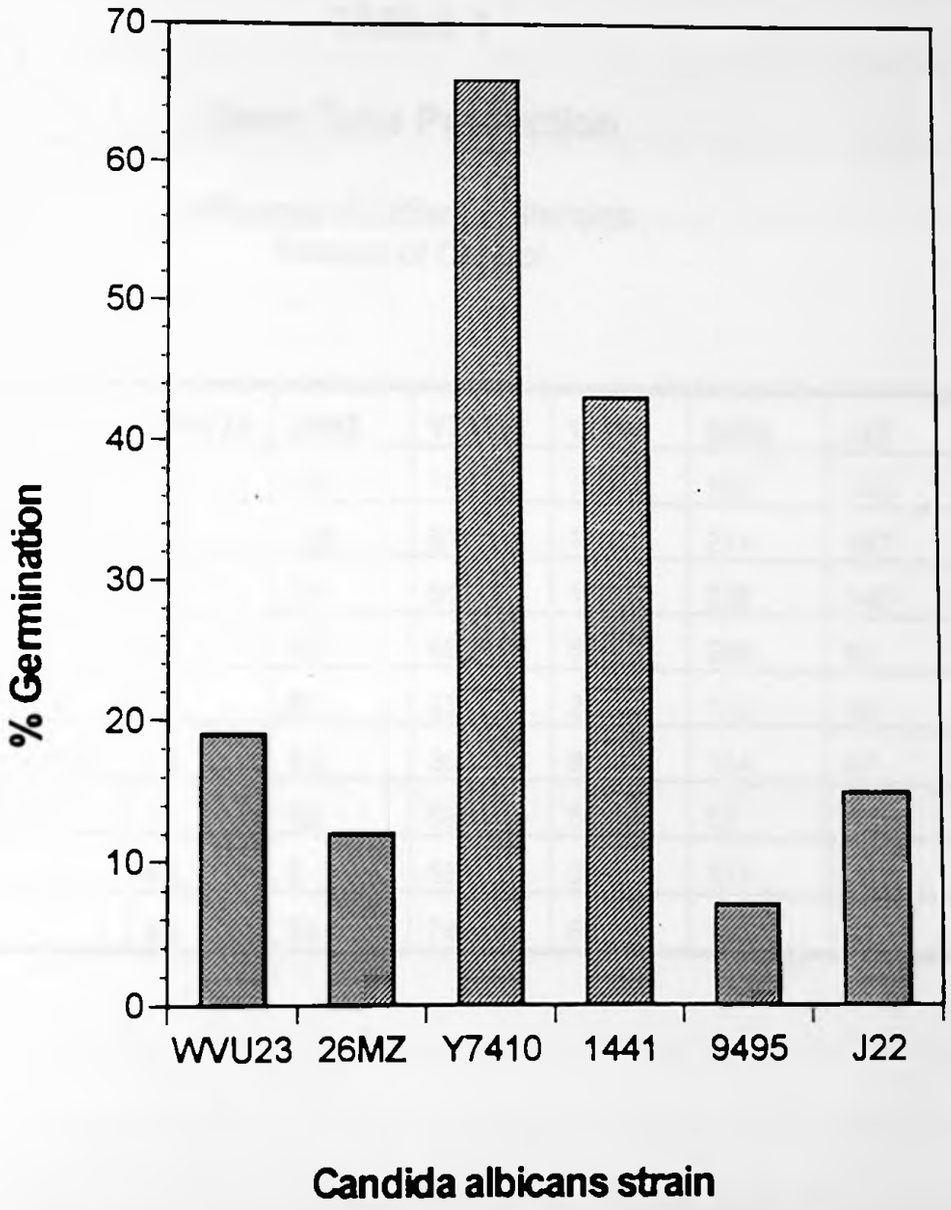


TABLE 1

Germ Tube Production

Influence of Different Steroids
Percent of Control

Hormone	WVU23	26MZ	Y7410	1441	9495	J22
control (1% EtOH)	100	100	100	100	100	100
estradiol	42	158	91	119	214	187
cholesterol	47	75	61	119	229	140
testosterone	42	83	68	65	285	67
progesterone	32	67	23	23	142	60
hydroxycortisone	26	50	30	9	114	67
prednisone	11	42	53	58	57	13
tamoxifen	63	8	55	33	171	107
DES	94	58	74	86	143	47

Four (26MZ, 1441, 9495, J22) of the six strains and cholesterol stimulated three of these strains (1441, 9495, J22). Strain 9495 showed an increase in germination with every compound except prednisone. These were such diverse chemical compounds with very different structures it is likely that a general, nonspecific mechanism is at work in this stimulation. It is interesting that the only compound the inhibited germination in all the strains was prednisone. All of the other compounds stimulated at least one strain.

Although estradiol did not stimulate every strain to produce more germ tubes, it did affect four strains. This aspect of *C. albicans* response to estradiol has some interesting trends that need to be investigated further.

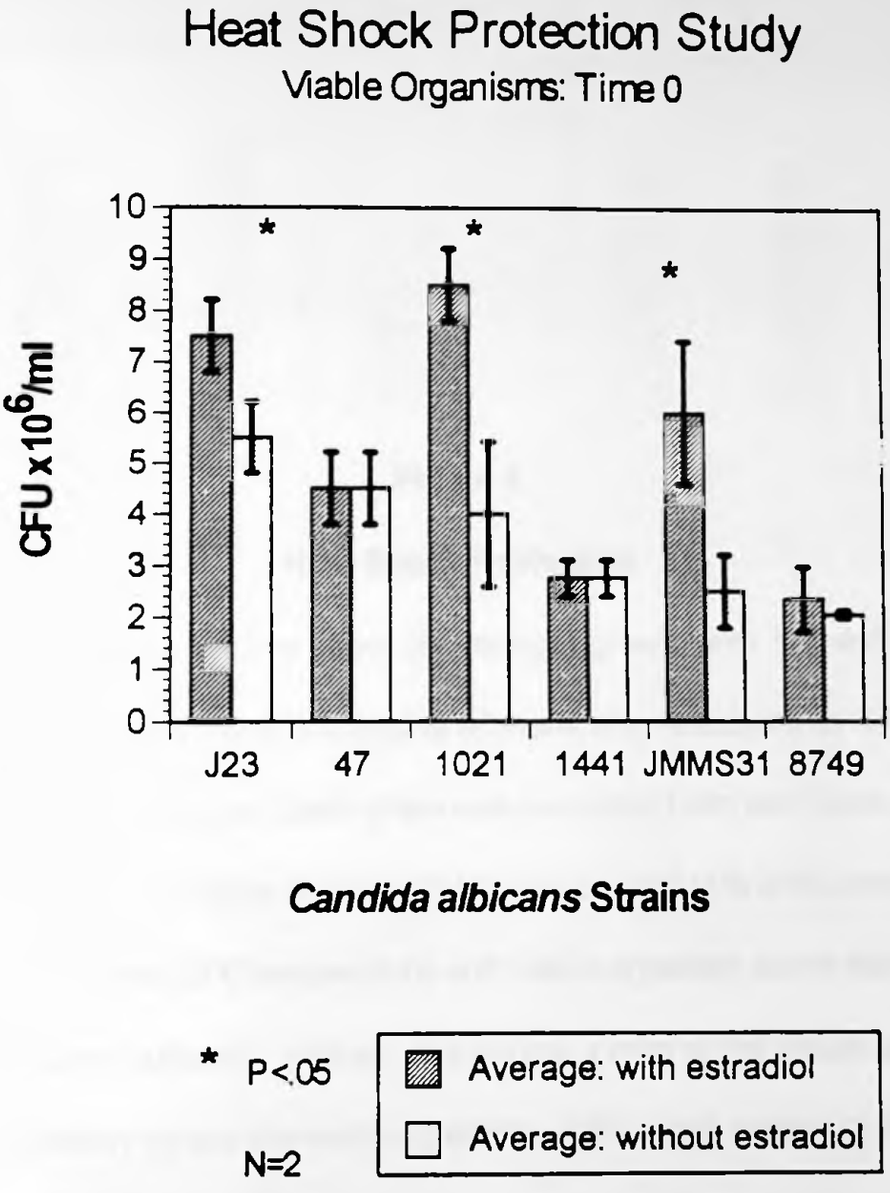
HEAT SHOCK PROTECTION

The culture collection was screened for growth stimulation or inhibition by 17β-estradiol in Sabouraud agar plates by measuring colony size (Gujar, in press). Based on these results, six strains were selected that were stimulated (J23, 47), not affected (1021, 1441), or inhibited (JMMS31, 8749) by estradiol and tested for survival of heat shock after exposure to estradiol. This experiment was performed twice. Figure 7 shows the difference in growth between the strains and the effect estradiol has on their growth. The values reported in this figure reflect the overnight growth at time 0. Estradiol significantly increases the growth of three strains, J23, 1021, JMMS31. Strains 47, 1441, and 8749 had the same growth with and without estradiol. Figure 8 shows the viable organism counts (CFU/ml) after fifteen minutes at 22°C, 37°C, and 50°C as a ratio of the final concentration to the

Figure 7

Viable organisms at time 0

Six strains were inoculated in to YCB+ medium, with 17-estradiol or with ethanol (1% final concentration), grown overnight at 25oC with shaking. Serial dilutions of the well-mixed cultures were performed and inoculated onto Sabouraud's agar at time 0 of the experiment. This study was repeated twice.


Heat Shock Protection Study
Viable Organisms: Time 0
CFU x 10^6/ml
10
9
8
7
6
5
4
3
2
1
0
*
*
*
J23
47
1021
1441
JMMS31
8749
Candida albicans Strains
* P<.05
N=2
Average: with estradiol
Average: without estradiol

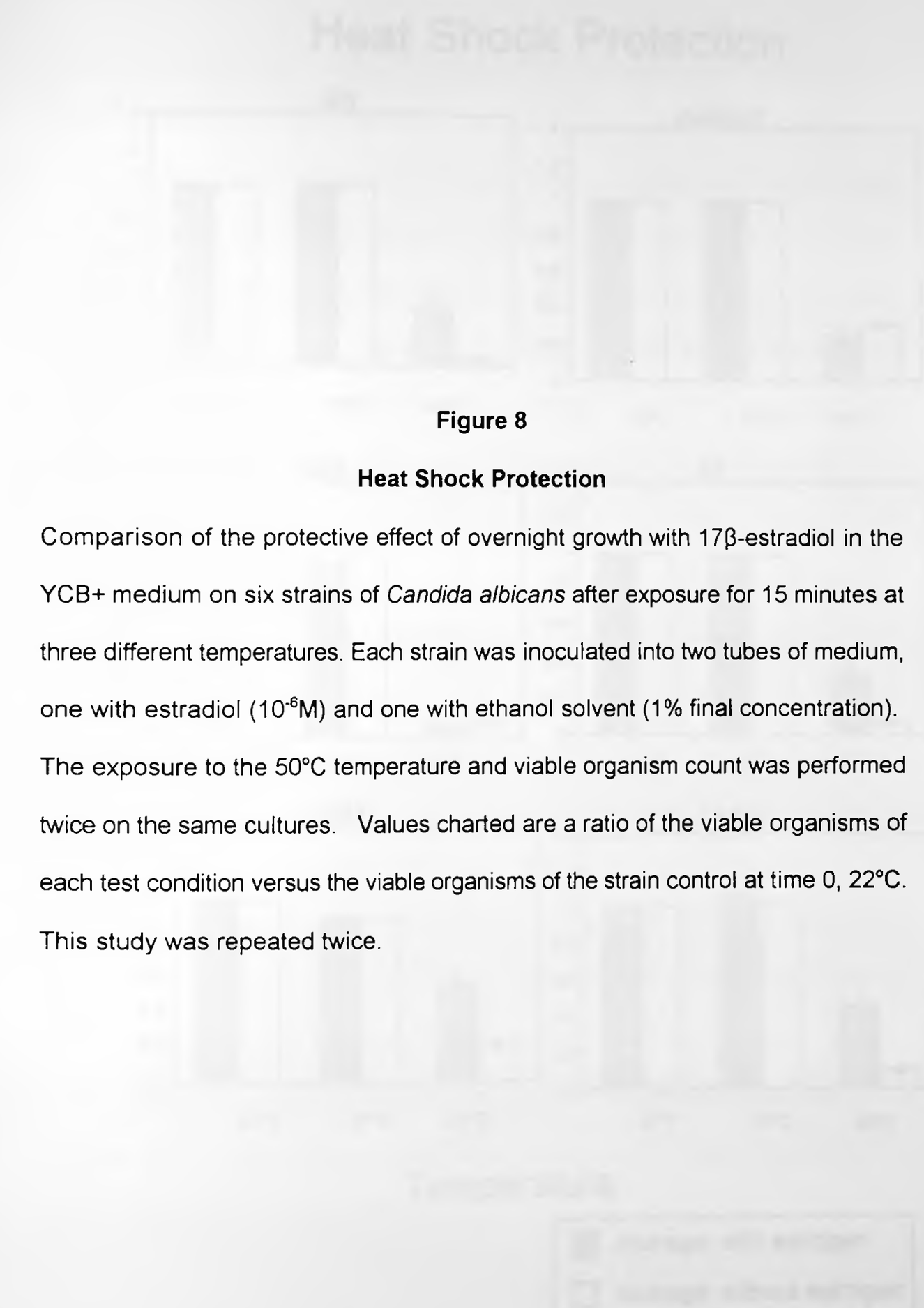
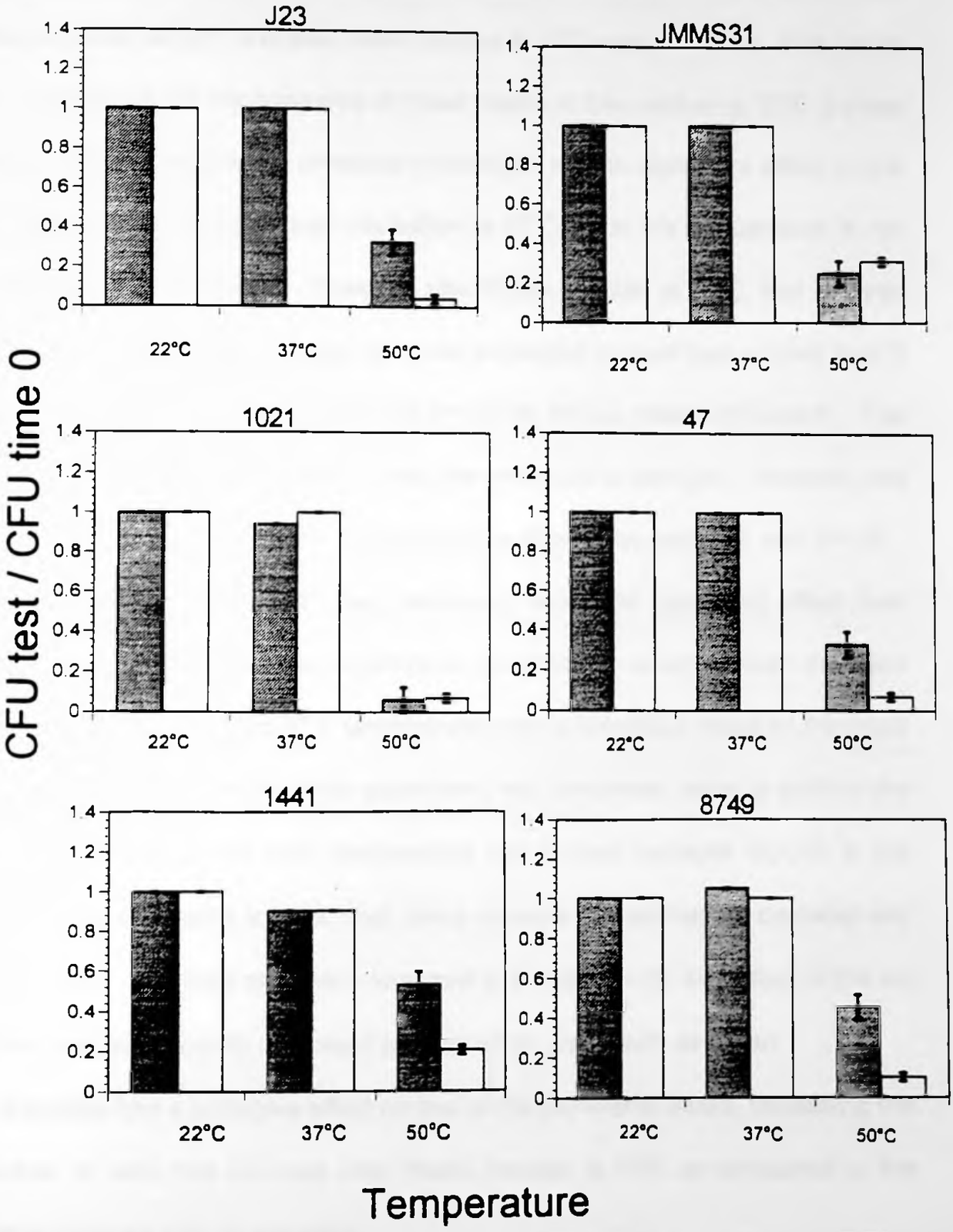


Figure 8

Heat Shock Protection

Comparison of the protective effect of overnight growth with 17β-estradiol in the YCB+ medium on six strains of *Candida albicans* after exposure for 15 minutes at three different temperatures. Each strain was inoculated into two tubes of medium, one with estradiol (10^{-6}M) and one with ethanol solvent (1% final concentration). The exposure to the 50°C temperature and viable organism count was performed twice on the same cultures. Values charted are a ratio of the viable organisms of each test condition versus the viable organisms of the strain control at time 0, 22°C. This study was repeated twice.

Heat Shock Protection



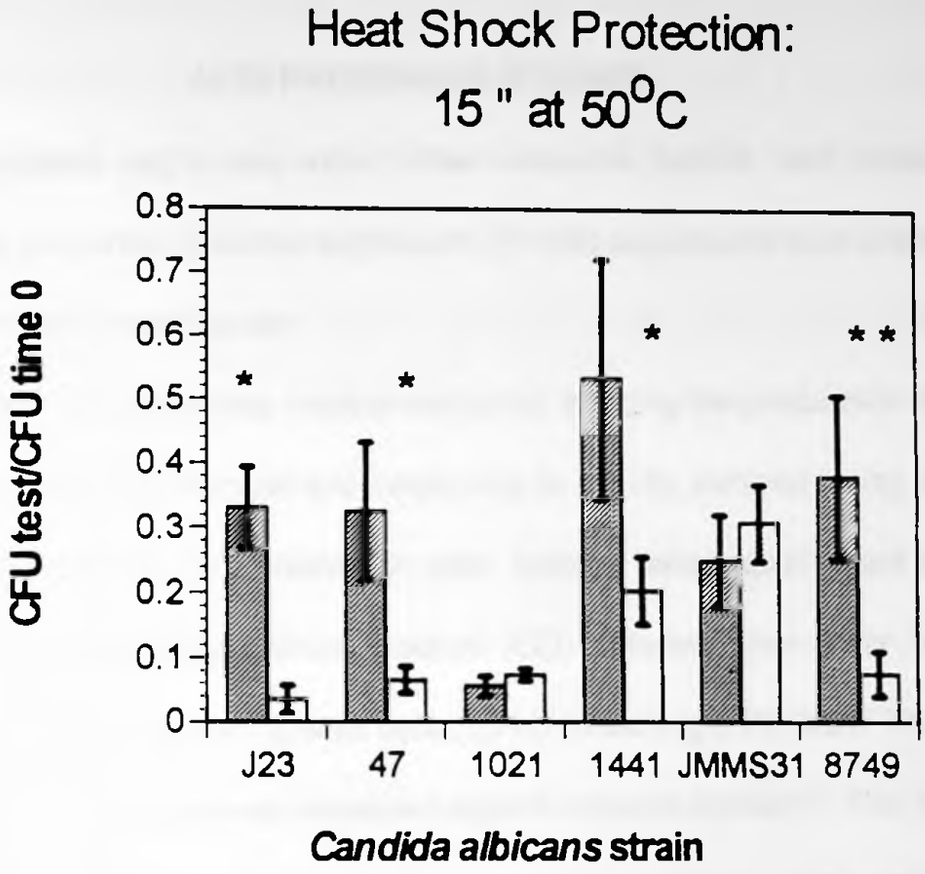
concentration at time 0. The CFU counts of the controls at time 0 at 22°C, after fifteen minutes at 22°C and after fifteen minutes at 37°C were the same. This would be expected as the doubling time of these strains in this medium at 22°C is three hours. Prior growth in the presence of estrogen had no significant effect on the survival after a fifteen minute incubation at 37°C since this temperature is not usually a lethal challenge. However, after fifteen minutes at 50°C, four cultures which had been exposed to estrogen had increased survival that ranged from 2 times (8749) to 14.5 times (J23), with $P<.05$ for all four strains (Figure 9). The mean survival increased about 4 times after exposure to estrogen. However, two strains (1021, JMMS31) were not significantly affected by estradiol, with $P>.05$. One interesting strain, 1441, had previously shown no significant effect from exposure to estradiol but was significantly protected by estradiol when thermally stressed. As the 22°C and 37°C temperatures had no detectable effect on the yeast strains, the 50°C portion of the experiment was performed twice to confirm the survival counts. The 50°C temperature was chosen because reports in the literature have used it to elicit heat shock proteins in mammalian, bacterial and yeast cells. The total data were analyzed and had a $P<.01$ since four of the six strains had significantly increased survival when grown with estrogen.

Estradiol had a protective effect on four of the six strains tested, increasing the number of cells that survived after fifteen minutes at 50°C as compared to the control cultures with no estradiol.

Figure 9

Comparison of Heat Shock Protection @ 50°C

Six strains were grown overnight, with and without 17β-estradiol in YCB+ medium, then exposed to 50°C for 15 minutes. Calculated as a ratio of the number of viable organisms (CFU/ml) of the test to the number of viable organisms (CFU/ml) of the control (time 0, 22°C). This study was repeated twice.



* P < .05

** P < .005

N=2

Average with estradiol

Average without estradiol

ACID PROTEINASE STUDIES

Since estradiol might also affect other virulence factors, acid proteinase secretion was examined. Estradiol significantly ($P<.05$) suppressed acid proteinase secretion per cell in each strain.

Development of a sensitive, reliable method for inducing the production of acid proteinase in *Candida albicans* and measuring its activity involved trying several different approaches. One method in petri dishes relied upon spent culture supernatant of chemically defined medium (CD) diffusing from wells cut into agarose (1% in 0.01M sodium acetate buffer, pH4) containing 0.1% BSA. The zone of clearing around the wells was measured against a pepsin standard. This method proved to be too insensitive. Other, more quantitative, methods using a solution of 1% BSA as the substrate were tried unsuccessfully. Research of the literature revealed that a pH>6.0 and any source of nitrogen, other than whole protein or a minimal amount of amino acids in the culture medium, suppresses the production of the acid proteinase. Even when ammonium sulfate was deleted from a chemically defined, minimal medium (CD) and BSA was added, no enzyme was produced. Induction of the enzyme was finally achieved with yeast carbon base (YCB) plus 0.2% BSA and 48 hour incubation at 30-35°C, with shaking (Milewski et al, 1994). The vitamins in YCB, and lack of nitrogen sources seem to be key elements. The assay for enzyme activity is described in the Materials and Methods section.

The culture collection was screened by this method to find strains that could be induced to secrete acid proteinase. Four strains were found that consistently secreted acid proteinase, though at different levels (J23, JMMS31, 7947, Y7410). Four different experiments were performed and analyzed. Since previous work had demonstrated an estradiol had an effect on growth (Figures 1,4,7), viable organisms were counted at the end of the incubation period. When viable cell concentrations were analyzed (Figure 10), estradiol had significant effect on the growth of two strains. Strain J23 was stimulated and strain 7947 was inhibited by the estradiol. Correction for different growth rates was made by dividing the enzyme units (enzyme activity defined as 1 Unit = ΔA_{280} of 0.1/ 30 min.) by the CFU $x10^7$/ml taken at the end of incubation to obtain the amount of enzyme produced by each viable cell. Acid proteinase, as expressed in enzyme units per viable organism, was significantly suppressed by the presence of estradiol in the inducing medium with all four strains (Figure 11). This calculation was analyzed with paired comparisons in the student-T test and each strain showed significant suppression of acid proteinase secretion when grown with estradiol, with $P<.05$. The overall significance across the strains was $P<.0001$. Since the growth of strain 7947 was suppressed with estradiol, the reduced enzyme levels in this strain may be a reflection of the reduced growth.

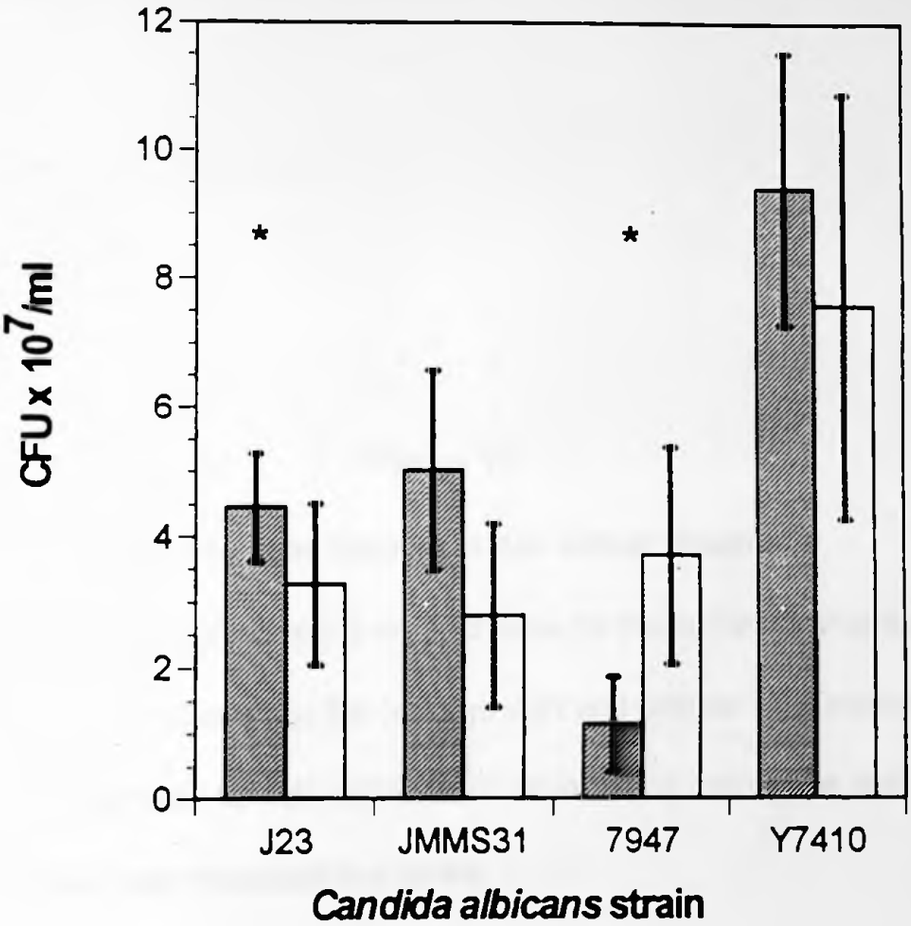
Other research in this laboratory inoculated *C. albicans* strains on casein-containing agar plates and measured zones of clearing (Foltman et al, 1985; Castillo, personal communication). Many more strains showed significant clear

Figure 10

Viable Organisms in the Acid Proteinase Study

Comparison of the growth rates of each *Candida albicans* strain after overnight growth in YCB+ medium, with 17β-estradiol or with ethanol (1% final concentration) at 37°C with shaking. Viable organisms were measured by colony-forming-units (CFU/ml) counted after serial dilutions were inoculated onto Sabouraud's agar. This study was repeated four times.

Acid Proteinase Study: Viable Organisms



* P < .05

N=4

Average with estradiol

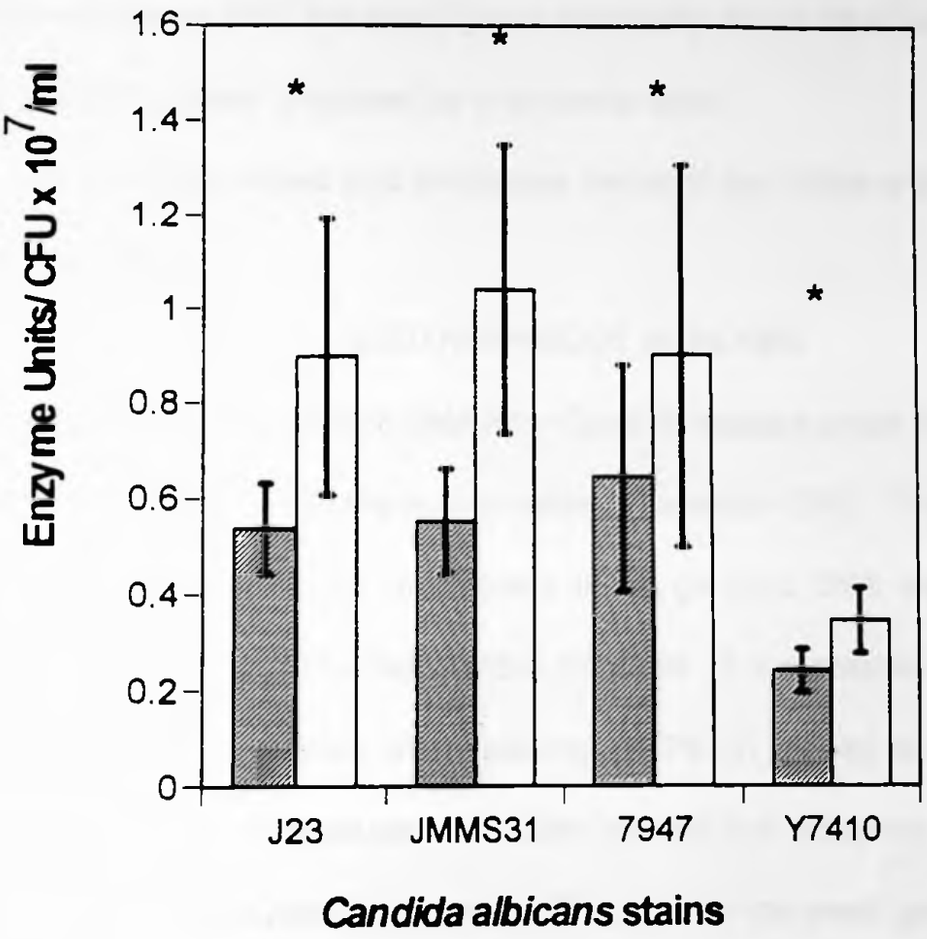
Average without estradiol

Figure 11

Acid Proteinase Secretion per Viable Organism

Enzyme units per CFU calculated to compensate for the variability in growth rates between the strains and between the cultures with and without 17-β estradiol. See Materials and Methods for full explanation of inducing conditions and enzyme assay. This study was repeated four times.

Acid Proteinase per Viable Organism



* P < .05

N = 4

With estradiol

Without estradiol

zones than produced enzyme in the Milewski method. However, since the medium pH was not measured and was very likely to be over 6.5, and the incubation temperature was 37°C, the clearing was most likely due to other proteases and not the acid proteinase proposed as a virulence factor.

Estradiol decreased acid proteinase secretion per viable organism in all four strains tested.

SOUTHERN BLOT ANALYSIS

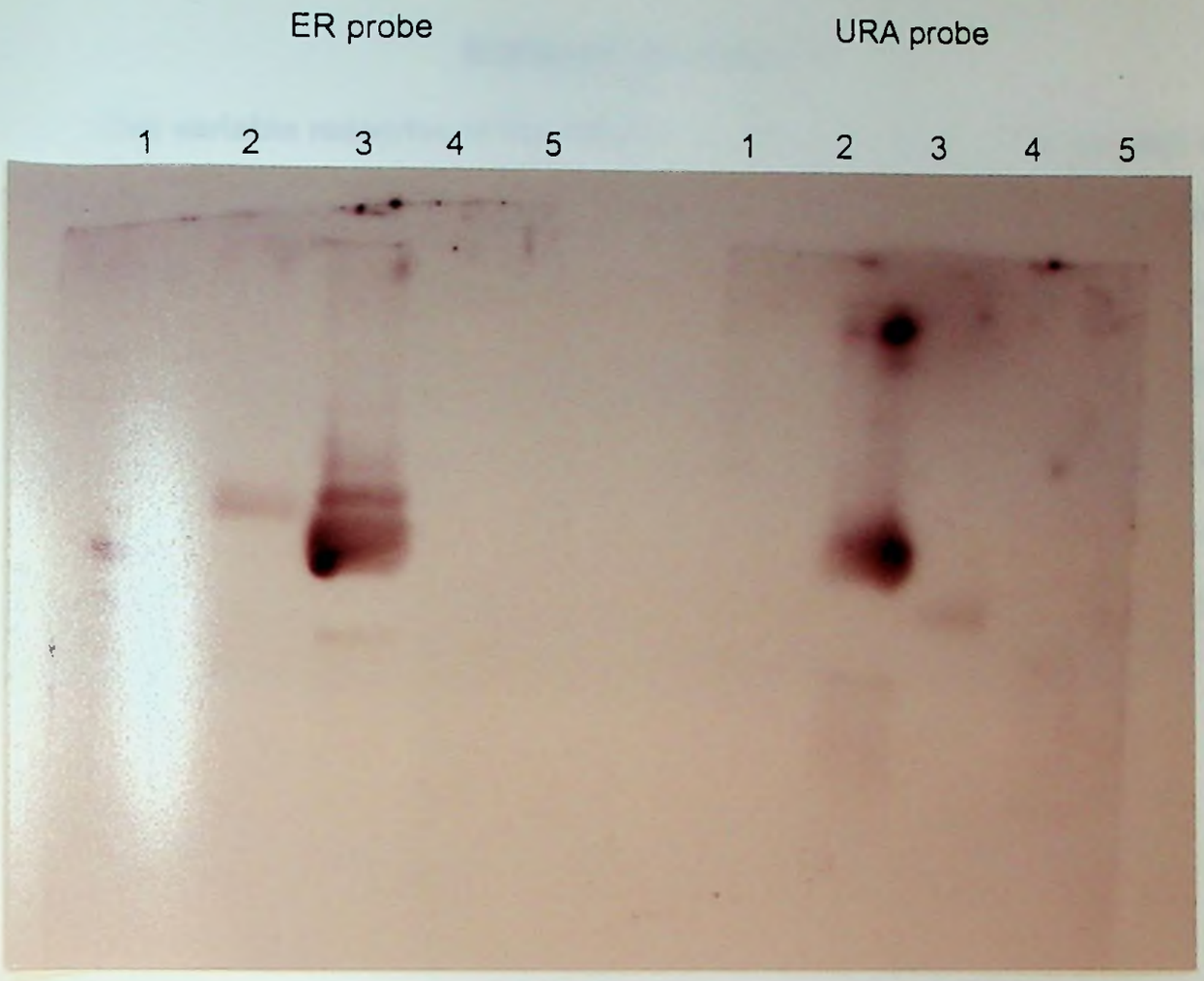
EcoRI digested genomic DNA from *Candida albicans* strain 9495 was probed with a 1.9kb fragment of the human estrogen receptor (ER). The CaURA control probe hybridized to a 3.0 kb fragment of the genomic DNA, the whole original plasmid (1041), and the BamHI/Xbal fragment of the plasmid. The ER probe hybridized to the purified, whole plasmid (YEPE10), as well as to the XhoI/SacI fragment from the digested plasmid. After low and high stringency conditions were used, very slight hybridization of the ER probe to the yeast genomic DNA was found. Cloning of the ~2.0kb fragment of yeast DNA that showed slight hybridization was attempted with no success. PCR was performed on *C. albicans* genomic DNA, using primers based on conserved regions of the human ER, chicken ER and fish ER that defined a 800bp conserved region. No product was made with any of the concentrations of magnesium chloride (1,2,3,4 mM) used.

The genetic studies undertaken here did not find evidence of a structural homolog for the human estrogen receptor in the genomic DNA of *Candida albicans*. Investigation for a functional homologue should continue.

Figure 12

Southern blot of *C. albicans* genomic DNA

EcoRI digested genomic DNA was probed under high stringency conditions with a 600bp BamHI/Xbal fragment of the CaURA3 gene as a control probe and a 1.9 kb Xho/SacI fragment of the human estrogen receptor (hER) as the probe for a structural homolog of the estrogen receptor. Chemiluminescence was used to detect hybridization.



Lane 1: molecular weight markers, λ phage digested with HindIII

Lane 2: plasmid 1041, containing CaURA, digested with BamHI and XbaI

Lane 3: plasmid YEPE-10, containing hER, digested with XhoI and SacI

Lane 4: genomic DNA of *E. coli*, digested with EcoRI

Lane 5: genomic DNA of *C. albicans*, digested with EcoRI

SUMMARY OF RESULTS

The variable response of the different *C. albicans* strains to 17β-estradiol is shown in Table 2. Some strains, such as J23, showed stimulated growth with estradiol under all conditions. Other strains, such as 9495, showed stimulated growth with estradiol only in some of the conditions and this strain also produced germ tubes more with estradiol. No response to estradiol was the distinguishing trait of such strains as 1441, although estradiol did have a protective effect on this strain in the heat shock experiment. This research indicates that response to estradiol depends upon the strain examined and the characteristic measured.

TABLE 2

Response to 17β-estradiol

Strain	Growth Rate CD 37°C	Protein: Growth YNB 37°C	Protein: Production	Germ Tube Production 37°C (1)	Heat Shock: Growth YCB+ 25°C	Heat Shock: Survival 15"@ 50°C	Acid Protein-ase: Growth YCB+ 30°C	Acid Protein-ase: Secre-tion	Growth: Colony Size Sab 37°C
J23					+	+	+	-	+
JMMS 31					+	0	0	-	-
7947							-	-	+
Y7410		0	0	0			0	-	+
WVU 23		-	+	-					0
26MZ		0	0	+					-
1441		0	0	0	0	+			0
9495	++	-	+	+					-
J22		-	+	+					+
47					0	+			+
1021					+	0			0
8749					0	+			-

\+ significant increase, $P<0.05$

0 no significant effect, $P>0.05$

\- significant inhibition, $P<0.05$

(1) + germination above strain control, 0 : germination the same as strain control,
- : germination less than control

DISCUSSION

GROWTH

We demonstrated that estrogen significantly stimulates growth of some but not all strains of *C. albicans*. The differences may be due to differences in growth conditions, such as the medium used, and/or to intrinsic differences among the yeast strains. Some of the most noticeable differences in growth are between the study done on Sabouraud's agar, which measured total colony mass and the broth cultures, which measured CFU/ml. Some of the strains were consistently stimulated (J23), some were stimulated in broth and suppressed in the plate assay (JMMS31) and some were not significantly affected in any system (1441). Better access to air in the plate study may be an advantage for some strains, although the broth cultures were also aerated.

Skowronski's work demonstrated that some *C. albicans* strains did not constitutively express high levels of estrogen-binding protein (EBP) and that when these yeasts were grown in yeast-nitrogen broth (YNB) medium, levels of cytosolic EBP were barely detectable. After growth in Sabouraud's-dextrose medium, these strains expressed more EBP but even this changed during the growth cycle, with peak levels during rapid growth (Skrowronski and Feldman, 1989). This might partially explain the variety of growth responses some strains demonstrated in our study.

17β-estradiol (1,3,5(10)-estriene-3,17beta-diol) may have its stimulatory effect by directly affecting signaling pathways in *C. albicans*. It would be interesting to

investigate the effect steroidal hormones have on the pathways in *C. albicans* that use cAMP and adenylate cyclase. Mammalian hormones may mimic endogenous steroid compounds involved in metabolic control or may induce a pathway that affects *C. albicans* (Baker, 1990). Given the highly conserved dehydrogenases involved in steroid metabolism that are found in microbes and mammals, it is possible that mammalian steroids are degraded by yeast and that the breakdown products are the active compound(s) in *C. albicans* (Baker, 1991; Baker, 1992). Future work in this area would measure the adenylate cyclase mRNA and cAMP levels in *C. albicans* after exposure to estradiol.

Work in this laboratory looked at the effect the flavinoid genistein has on cell growth. In chemically defined (CD) medium, supplemented with genistein after glucose deprivation, some strains showed profoundly increased growth over the control and increased growth over the estrogen supplemented medium as well (Yijian Fu, personal communication). Future work in this area should investigate whether radiolabelled genistein binds the EBP of *C. albicans* and whether estradiol competes for the same binding site. Alternate pathways, such as the cAMP cascade versus signal transduction via the EBP, could be chemically blocked to find the mechanism genistein uses to increase growth. Genistein is known to bind the hER and transactivate estrogen responsive genes in breast tumor cell cultures. Since estrogen has an effect on some strains of *C. albicans* but no estrogen responsive genes have been identified yet, genistein may help elucidate the regulation of growth in *C. albicans*.

The 'classical' signaling pathway which uses a steroid receptor binding to a hormone responsive element for transactivation may not be involved in the effects estradiol has on *C. albicans*. Another possibility is that estradiol, or a metabolite, is substituting for a nutrient or a vital precursor of a structural component such as ergosterol, which is a part of the cell membrane. Experiments that investigated the effect of estrogen and progesterone on the bacteria *Bacteriodes melaninogenicus* found that either hormone could substitute for vitamin K, an essential growth factor. The mammalian hormones appeared to interact with the fumarate reductase system in the bacteria (Kornman and Loesche, 1982). If estradiol is a substitute for a compound in the elaborate sterol metabolism of the fungus, one would expect that cholesterol might also substitute and have the same effect as estradiol. Although cholesterol did increase growth and biomass over the control, it was not as dramatic as with estradiol. The lag period before stimulation could be explained if a metabolite of estradiol is the molecule affecting growth and metabolism is at a minimum level because of the minimal defined medium.

Substituting for a growth factor would also help explain the differences observed with Sabouraud agar, which uses peptone, a complex, undefined and variable component. Some strains might not need the nutrient or growth factor substitute when grown on Sabouraud or might not be able to metabolize the hormone very efficiently. Other nutrient levels may be responsible for the lag time seen in the growth dynamics study. Repeating the study with sequential dilutions of Sabouraud broth should determine if the nutrient level is contributing to the growth lag. There

is also the possibility that estradiol is acting synergistically with some growth factor in the Sabouraud's medium since peptone is a component of the medium and peptone can be quite variable depending on the source of the protein and the enzymes used to digest it.

Though there is no evidence that synergism or cross-talk is involved in *Candida albicans* response to mammalian hormones, no one has used combinations of hormones or hormone metabolites and looked at the effect. Estradiol may have its effect on the growth of *Candida albicans* through the estrogen-binding protein (EBP) or cross-talking with a growth factor pathway. The EBP shows high affinity for estradiol and yet bears homology to oxidoreductases and not to steroid receptors (Saito et al, 1991; Stott et al, 1993). Estradiol has an inhibitory effect on the oxidoreductase enzyme activity (as measured by NADPH oxidase activity) of the EBP so that estradiol may have an effect on the energy metabolism of the cell (Madani et al, 1994). Researchers found that EBP levels vary between *C. albicans* strains and during the growth cycle. Some strains demonstrated tight regulation of estradiol binding depending upon the medium that the organism is grown in, while other strains produced EBP at constitutively high levels (Skrowronski and Feldman, 1989).

Follow-up experiments to our growth studies should screen more strains to come up with at least two strains each that are either consistently stimulated, not affected, or inhibited by estradiol in a defined but not minimal medium, like yeast carbon base (YCB). Then radioactively-labeled hormones could be used to see if the

hormones are incorporated into the cell membrane. The many known mutants in sterol synthesis, which are nystatin-resistant, could be used to pinpoint the part of the ergosterol pathway that incorporates the labeled hormone. The laboratory culture collection could also be screened for nystatin resistant mutants for use in this experiment, though the exact defects would not be defined.

It may also be possible that the estradiol-treated cultures grow faster initially, exhausting the nutrients available, and so start to die off by day four. This experiment should be repeated, using YCB medium, and taking measurements every two hours for the first day, and everyday for a week thereafter to more fully document what is occurring. Correlating the viable organism count with the absorbance at 650nm would show differences between the number of live organisms and the total number of cells, alive and dead. It could be that estradiol has a deleterious effect upon viability after several days, as seen in the extracellular protein study.

Growth is not the only attribute of *C. albicans* that can be affected by mammalian hormones. Some researchers have reported that 17β-estradiol increased protein synthesis in *C. albicans*. This led us to investigate various phenotypic aspects of different *C. albicans* strains in relation to their reaction to estrogen.

PROTEIN

These total extracellular protein studies failed to follow any significant pattern across the six strains tested. The two strains that did show significant increases in protein secretion when grown with estradiol indicate that at least some strains are

affected by the hormone. Research by other laboratories reported an increased rate of protein synthesis in one strain of *C. albicans* after exposure to 17β-estradiol (Frey et al, 1988, 1988). Given the differences found within the culture collection in our laboratory, it is quite possible the strain used in the other laboratory has a different response than the ones used in our study.

Comprehensive evaluation of growth and extracellular protein production in the presence of estradiol under various conditions of protein concentration, pH, aeration, etc. should be employed to provide a more complete picture of estradiol's effect on protein secretion. If differences are seen in the total protein, with or without estradiol, protein electrophoresis on the concentrated culture supernatant might reveal any new proteins that are produced under the influence of estradiol. If estradiol's effect is through overall metabolism it is more likely that overall production is increased.

New or increased levels of constitutive proteins might indicate an increase in such virulence factors as adhesins, which are expressed on the surface and secreted enzymes, such as acid proteinases or proteins that contribute to morphogenesis or pathogenesis.

GERMINATION

As found with the growth studies, strains vary in displaying this phenotype. The tendency to germinate and the responsiveness to steroidal hormones differed greatly between strains, although many instances of germ tube inhibition were noted.

An attempt was made to remove any hormonal effects the germ tube induction medium might exert by using somewhat purified sources of protein (peptone and BSA). The traditional induction medium is serum which can contain steroid hormones and growth factors. Germination occurred usually within 30-60 minutes when the blastospores are placed in medium containing 7% serum. It usually took two hours for germination to start in the semi-defined medium used in these studies. Inoculating a control with no hormone should compensate for any endogenous hormonal effect the serum exerts on germination. But germination normally occurs so rapidly in serum that any acceleration would be hard to document. Alternatively, the experiment could be done with serum treated with activated charcoal before using it in the induction medium. Other researchers studying germination have used an homogenized kidney extract (Kinsman, 1988), which would be subject to the same objections as the serum, or the original Lee's synthetic medium (LSM) (Lee et al, 1975), a defined medium which contains amino acids, and avoids those objections (Williams et al, 1990).

For statistical significance this study needs to be replicated. There are significant differences between the strains in the rapidity of germ tube production, overall germination rate and cohesiveness of the germ tubes. Some strains are such rapid germinators and so adhesive they form clumps that make accurate germ tube counting difficult. Continuous video analysis through the microscope might be able to overcome some of these problems. However, unless a heated microscope

stage were available, the incubation temperature would have to be changed from 37°C to 22°C, which will slow the germination and may prevent it in some strains. Investigating the effect of overnight growth in a medium with the hormones and then incubation in hormone-free GTI medium might be helpful also.

To investigate the possibility of synergism between the hormones, each hormone could be paired with each of the others and added to the germination induction medium. Hormones that prove significantly effective either in suppressing or stimulating germination could be titrated to determine the minimal effective concentration. The concentration of 10^{-6}M for the hormones was used in these assays because it is comparable to concentrations commonly used in the literature.

HEAT SHOCK

Prior incubation with estrogen appears to have a protective effect on some strains of *C. albicans* when they are subsequently exposed to thermal stress (Figure 9). This study was prompted by numerous reports documenting the protective effect estrogens exert on mammalian cell survival from stress (Billig et al, 1993; Wang and Phang, 1995; Behl et al, 1995). Even though the 50°C temperature used to stress the yeast would not normally be encountered by the microbe during the colonization or infectious process, the response elicited would be similar to the response to any other stress, such as oxidative (from neutrophils) or nutritional (from host iron-sequestering proteins). Microbes also often employ

relevance to those conditions in vivo. This finding illustrates some of the continuing pitfalls of studying putative virulence factors in vitro and the failure to have any means for controlling for the adaptations that may occur among laboratory strains repeatedly cultivated on artificial medium.

GENETIC STUDIES

Estradiol's effect on *C. albicans* is sometimes stimulatory of growth but suppressive of acid proteinase secretion. We decided to probe the genome of one *C. albicans* strain that had shown stimulation of growth. Southern blot analysis of the genome of strain 9495 was made with a conserved fragment of the hER. These studies gave no evidence for a genetic analog of the human estrogen receptor (hER) in the genome of *Candida albicans* but a functional analog is not precluded. Other estradiol responsive *C. albicans* strains could also be probed for a structural homologue.

The *Saccharomyces cerevisiae* expression system using an ubiquitin-estrogen receptor (ER) fusion protein and vitellogenin estrogen responsive element (ERE) reporter plasmid (McDonnell et al,1991) could be adapted to screen the *Candida* genome for a functional ERE. The *S. cerevisiae* system uses the plasmid YRpE2, containing the vitellogenin estrogen responsive element (ERE), as the reporter plasmid, and the plasmid YEPE10, containing the human estrogen receptor (hER) as the expression vector. The fact that this expression-reporter system works in a yeast indicates that the accessory proteins necessary for receptor-ligand and receptor-DNA interaction exist and function with the mammalian elements in yeast.

Hsp90 is highly conserved across species and the other receptor associated proteins may have homologs in yeast. The only estrogen-binding protein to be found in *Candida albicans* has no homology to the human ER but to flavoproteins and oxidoreductases. This does not preclude the existence of an ERE-like promoter in *Candida*.

Other types of genetic studies should be done, such as DNA fingerprinting with the moderately repetitive sequence Ca3 to determine if estradiol responsive strains are genetically similar. DNA fingerprinting involves purifying the genomic DNA of an organism, cutting it with restriction endonucleases, and probing it in a Southern blot with a probe made from a repetitive sequence (like a ribosomal RNA sequence). The pattern of hybridization will be distinctive for each strain. Recently isolated strains from clinical specimens of symptomatic women should also be fingerprinted and their phenotypes defined. These characteristics should be retested after repeated in vitro subculturing to monitor any changes that occur.

FUTURE PROJECTS: ADHERENCE AND iC3b

One area not addressed in this study is adherence. Adherence of a microorganism to the host is usually the first step in the infectious process. Microbial adhesins not only recognize specific eukaryotic tissues, they may initiate invasion by a pathogen (Schlesinger et al, 1990; Saukkonen et al, 1992; Isberg, 1991). They have inherent abilities to activate, subvert or exploit the host defense systems and can direct host cell functions like cytokine induction from endothelial cells and leukocytes, and cytoskeletal rearrangement of macrophages

(Hoepelman, 1992; Isberg, 1991; de Man et al, 1989; Westerlund et al, 1992). Commensal organisms like *C. albicans* have evolved mechanisms for adherence to and maintenance of the host tissues without triggering a response from the host that would eliminate the organism.

The complicated realm of *C. albicans'* adherence to host surfaces has been reviewed, outlining the various host factors and attributes of *C. albicans* that contribute and change this property. Many of the assays for adherence involve meticulous control of many environmental, host tissue, and strain conditions as well as somewhat subjective microscopic observation. The more recent studies of the iC3b receptor analog expressed on the surface of *C. albicans* leads to interesting speculation and opens many possibilities for further research that avoids the subjectivity problems because flow cytometry can be used for measurements.

Hostetter's work demonstrating the upregulation of expression of the iC3b receptor by *C. albicans* when the glucose concentration is increased, leads to speculation that perhaps other environmental signals, like hormones, affect the level of expression of this integrin analog. As stated before, diabetics, with their increase in blood levels of glucose, and pregnant women, with their increase in levels of estrogen and progesterone, are predisposed to candidiasis (Marvel et al, 1938; Odds, 1988a). If it is demonstrated that estrogen upregulates the expression of iC3b on *C. albicans* surface, then the levels of mRNA for iC3b receptor could be examined with Northern blots, using INT1 as a probe. That would determine if the

regulation is transcriptional. The regulation of CR3 in neutrophils is not transcriptional, as cytosolic stores are translocated when expression is increased.

SUMMARY

These studies have verified that estradiol stimulates growth in vitro of some *Candida albicans* strains, though the effect is not uniform for all strains under all conditions (Table 2). The phenomena such as extracellular protein production, germination and acid secretion were quite variable in the response to estradiol. Evidence from this study, coupled with the literature, indicates that when estradiol stimulates *C. albicans* it may be more through a metabolic mechanism rather than through a hormone receptor activating genes. The wide variety of characteristics displayed by *C. albicans* strains, without detectable changes in the genomic DNA, and the different responses to estradiol, between the strains and between experiments, can be explained only when further investigations look at messenger RNA levels. Phenotypic changes involve so many proteins and genes, only by demonstrating changes in the mRNA and protein products from key genes will a true picture emerge. Opportunity still exists to scrupulously investigate these phenomenon. Regardless of the mechanism through which stimulation occurs, mammalian hormones may affect *Candida albicans* cells.

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