

## Flow Cytometry of *Candida albicans* for Investigations of Surface Marker Expression and Phagocytosis

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**Abstract.** Several cytoplasmic virulence factors of *Candida albicans* are altered in the presence of estrogen and this fact may imply the existence of a global virulence regulatory system in this organism. The response of virulence-associated surface markers to estrogen, however, has not been studied. We exploited flow cytometry methods for assessment of the iC3b receptor analog and mannoproteins on 2 clinical yeast strains selected for their different rates of growth in the presence of estradiol 17 $\beta$ . Although, as expected, iC3b receptor analog expression increased in the presence of glucose, growth in the presence of estradiol did not increase the levels of iC3b receptor analog on either organism. Exposure to human serum caused massive conversion to mycelial growth, but cells examined by flow cytometry did not show increased levels of iC3b receptor analog expression, possibly due to inability of the flow cytometer to sample the mycelial forms of *Candida*. In contrast, estradiol increased expression of mannoproteins as evidenced by concanavalin A binding to yeast. This increase occurred in both yeast strains but was less pronounced with strain GT188, which also showed limited growth in estradiol compared to strain GT142. Effective phagocytosis by human neutrophils required exposure of yeast to human serum. Yeast grown in the presence of estradiol were ingested by human PMN but not at a significantly greater rate than yeast grown without estradiol. While flow cytometry appears to be useful in determining estrogen-enhanced concanavalin A binding to yeast, it probably does not reflect the surface markers on large mycelial masses. Consequently, the results of this study are applicable to *Candida* primarily in its yeast form. (received 18 December 2004; accepted 16 March 2005)

**Keywords:** yeast, candidiasis, estrogen, concanavalin A, virulence factors, surface markers, phagocytosis

### Introduction

*Candida albicans* interacts with human hosts in an exceedingly complex manner, displaying a wide range of interactions from colonization of mucosal surfaces without invasion, to symptomatic mucocutaneous infections, to systemic disseminated disease. The complexity of these host-microbial interactions is evidenced by the hundreds of research reports on this topic that appear in the medical literature each year, and yet it remains incompletely explained how this organism can change from commensal to pathogen within the same individual.

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One important aspect of host-parasite interactions is the way in which *Candida albicans* adapts to its environment by upregulating virulence factors at appropriate times. Previous research has demonstrated modulation of virulence expression in response to environmental cues [1-5]. The increased propensity of colonizing *Candida* to produce symptoms in pregnant women engendered particular interest in virulence factors affected by estrogen. Estrogen-sensitive properties include the growth rate of *Candida albicans* [6], morphologic change that favors mycelial growth [7], increased expression of methionine synthase [8], increased heat shock protein -90 production [9], and higher levels of CDR1 (Candida drug resistance gene) messenger RNA [10]. All of these factors have been associated with exposure to estradiol in specific fungal strains.

Because these seemingly disparate factors show responsiveness to the presence of estrogen during cultivation of *Candida albicans*, it is possible that other virulence factors not yet studied in relationship to environmental estrogens may also be influenced by estradiol and other steroids. We hypothesize that exposure of *Candida albicans* to estradiol may alter specific and non-specific surface markers on this organism such that interaction with tissues and phagocytic cells increases the organism's pathogenic potential. Surface properties such as cell surface hydrophobicity [11], mannosylated surface proteins [12], and expression of an analog of iC3b receptor [13] have also been associated with virulence, but regulation of their expression has not been studied with respect to estrogen exposure.

Flow cytometry is a powerful technique for probing the surface of cells, but has previously had limited application to studies of pathogenic microorganisms. It is reasonable to expect that flow cytometry could yield useful data on the surface properties of fungi. In this report we exploit flow cytometry methods for probing selected surface properties of *Candida albicans* and culture conditions that may alter these surface properties.

## Materials and Methods

**Organisms.** *Candida albicans* strains GT-142 and GT-188 were originally obtained as clinical isolates and were maintained on Sabouraud's dextrose agar at 4°C and transferred to fresh media at 3 mo intervals. Original stocks of these isolates were also kept at -70°C for long-term storage. Organisms recovered from -70°C showed similar responses to estrogen as isolates passaged and stored at 4°C. Unless otherwise noted, test organisms were grown overnight at 37°C in yeast nitrogen broth (YNB) with 28 mM glucose and 10% fetal calf serum. Composition of the growth medium was altered for some experiments to include the addition of 10<sup>-9</sup> M estradiol (1,3,5-10-estratriene-3-17β-diol, Sigma Chemical, St. Louis, MO). Since estradiol was dissolved in methanol, experiments involving addition of estradiol employed an equal volume of methanol for the controls. The volume of methanol added in such experiments was 1% of culture volume.

**CD11b/Mac-1 antibody staining.** After overnight growth as described above, yeast were washed in phosphate buffered saline (PBS), resuspended, and adjusted to 1.5 absorbance units (AU) at 600 nm in PBS with 1% bovine serum albumin (BSA). Phycoerythrin-conjugated CD11b/Mac-1 antibody (BD Biosciences, San Diego, CA) was added to yeast suspensions

in a microtiter plate and incubated at 4°C for 30 min in the dark. The optimal concentration of CD11b/Mac-1 (10% v/v) antibody was determined experimentally (data not shown). Following incubation, CD11b/Mac-1 stained cells were transferred to 1 ml of sheath fluid (Becton Dickinson, San Diego, CA) and analyzed by flow cytometry.

**Concanavalin A.** Concanavalin A conjugated with fluorescein isothiocyanate (FITC) was obtained from Sigma and used at a final concentration of 0.0125 mg/ml, which was determined experimentally (data not shown). Yeast were prepared as for staining with CD11b/Mac-1.

**FITC-Labeling of yeast.** Test organisms were grown as described above and washed with PBS. Washed yeast were harvested and resuspended in carbonate buffer (0.5 M, pH 9.5) and adjusted to 1.4 AU at 600 nm. One ml of the yeast suspension was added to 990 μl of a 0.01 mg/ml FITC solution (Flucka BioChemika, Switzerland) in carbonate buffer, and incubated at 37°C for 30 min in the dark. FITC-labelled yeast were harvested by centrifugation and resuspended in 1.5 ml of Hank's balanced salt solution (HBSS).

**Preparation of neutrophils.** Blood was obtained from a human volunteer who consented to this study, which was approved by our Institutional Review Board. Whole blood (10 ml) collected in EDTA-containing Vacutainer® tubes (Becton-Dickinson, Franklin Lakes, NJ), mixed with an equal volume of 3% Dextran T-500 (Pharmacia, Uppsala, Sweden) in saline and incubated at room temperature for 30 min. The top (leukocyte-rich) layer was aspirated into two 15 ml conical tubes and pelleted by centrifugation (10 min, 150 x G, 4°C). The supernatant was removed and the cells were resuspended in a volume of 0.9% NaCl equal to the initial volume of blood. An equal volume of Ficoll-Paque PLUS (StemCell Technologies, Vancouver, BC) was then pipetted underneath the leukocyte-rich solution, ensuring a clear division between the 2 layers. The tubes were centrifuged (40 min, 500 x G, 20°C) with no brake. After centrifugation, the top 2 layers as well as the cloudy monocyte layer were removed, leaving only the neutrophil/RBC pellet at the bottom of the tube. The residual red blood cells were lysed by the addition of ice-cold 0.2% NaCl for exactly 30 sec, followed by addition of an equal volume of 1.6% NaCl to restore tonicity. The cells were centrifuged (6 min, 150 x G, 5°C), and the process was repeated until the pellet was free of any visible residual red blood cells. The neutrophil pellet was resuspended in 1 ml of ice-cold HBSS and neutrophils were counted with a hemocytometer.

**Neutrophil phagocytosis of yeast.** In a 10 ml glass tube, neutrophils were mixed with FITC-labelled yeast in a 1:10 ratio of neutrophils to yeast to obtain a 1.5 ml final volume and placed in a 37°C shaking water bath (135 rpm). Immediately after mixing the neutrophils with the yeast, a 250 μl aliquot was removed and placed on ice in a flow cytometer tube containing 0.9% NaCl with 0.02% EDTA to arrest phagocytosis. At 15, 30, 45, and 60 min after the start of the reaction, 250 μl aliquots

were again removed and added to flow cytometer tubes containing 0.9% NaCl with 0.02% EDTA on ice. The samples were analyzed by flow cytometry gated for collection of neutrophils.

**Internalization assay.** To determine if yeast cells were either bound to the surface of neutrophils or internalized, the method of Busseto et al [14] was employed. In this assay, trypan blue is added to the yeast-neutrophil mixtures and internalized yeast are detected by fluorescence in the FL2-H channel.

**Flow cytometry.** Flow cytometric evaluation of yeast and neutrophils was performed using a BD FACScan flow cytometer with linear amplification of forward and side-scatter channels. Fluorescence channels 1 and 2 (FL1-H and FL2-H respectively) were monitored with logarithmic amplification. PE-labelled CD11b/Mac-1 antibody yielded fluorescence in the FL2-H channel and FITC-labelled concanavalin A was detected in the FL1-H channel. FITC-labelled yeast cells in the phagocytosis experiments were detected in the FL1-H channel and internalized trypan blue-labeled yeast were detected in the FL2-H.

As reported in a previous binding study [15], 2 parameters were evaluated for each experiment. Percent M1 was derived by placing a marker (M1) on the number of events versus fluorescence intensity histogram plot to indicate the percent of events having fluorescence above 99% of unstained cells (%M1). The second parameter recorded was the geometric mean channel fluorescence (GMCF) of all organisms detected in the sample; this indicates the overall fluorescence of the entire population. These statistics were generated by the program Win-MD1 using data files obtained from the BD FACScan instrument. To indicate the relationship between untreated and treated cells, the fluorescence of control cells was divided by that of treated cells to yield a fold-increase over control (FIOC).

## Results

**Surface iC3b receptor.** Two strains of *Candida albicans* were used in this study. One strain (GT142) was chosen because it showed increased growth in  $1 \times 10^{-9}$  M estradiol-17 $\beta$  (cell counts after overnight growth in estradiol were 1240% of growth in the absence of estradiol); the other strain (GT188) was chosen because it showed decreased growth in estrogen-supplemented medium (cell counts after overnight growth in estradiol-17 $\beta$  were 10% of the cell count in the absence of estradiol).

The iC3b receptor analog is a surface marker originally studied by Hostetter's group [13]. To test for its presence, yeast strains were reacted with CD11b/Mac-1 antibody and analyzed by flow

cytometry. As shown in Fig. 1, both yeast strains displayed significant binding of the CD11b/Mac-1 antibody. Unstained yeast showed some autofluorescence, so it was important to account for this background fluorescence in the data analysis. Approximately 75% of yeast cells exposed to CD11b/Mac-1 showed greater fluorescence than 99% of unstained organisms (%M1). Geometric mean channel fluorescence (GMCF) was 8-10 x greater for stained compared to unstained organisms. CD11b/Mac-1 antibody was not from the same clone as that used by Hostetter et al [13] (anti-Mol-1). While CD11b/Mac-1 antibody bound to yeast, it was not clear that CD11b/Mac-1 was truly straining the iC3b receptor analog that was the subject of Hostetter et al's study [16]. To strengthen evidence that CD11b/Mac-1 was detecting the iC3b receptor analog, the effect of yeast cultivation in different concentrations of glucose was examined. The anti-Mol-1 staining used by Hostetter et al [16] was reported to be increased when cells were grown in high concentrations of glucose or when cells underwent mycelial transformation. Hostetter et al's work [16] showed that 20 mM D-glucose had a 4-6 x increase in receptor expression over yeast grown in 20 mM L-glutamate (employed as a non-carbohydrate carbon source). Growth of test organisms in media with increased glucose concentration was therefore evaluated and resulted in a corresponding increase in receptor expression. Elevated glucose levels furnished increased levels of CD11b/Mac1 binding as shown in Fig. 2. It was therefore concluded that CD11b/Mac-1 was behaving in a manner similar to previous reports of anti-Mol-1 binding and was probably detecting the same surface protein that Hostetter et al [16] examined, namely the iC3b receptor analog.

Since virulence factors may be altered by environmental conditions, several characteristics including growth temperature, germination status, and serum binding were evaluated with CD11b/Mac-1 antibody. Previous research from our laboratory [15], in which flow cytometry was employed to quantify styrene bead binding to yeast (a characteristic associated with cell surface hydrophobicity) revealed that bead binding was increased in yeast incubated at 25°C, compared to that observed at 37°C. Yeast strains were grown in YNB

Table 1. Effect of growth temperature on CD11b/Mac-1 binding to *Candida albicans*.

Yeast strains	Incubation at 25°C without fetal calf serum			Incubation at 37°C with 10% fetal calf serum		
	%M1	GMCF	FIOC	%M1	GMCF	FIOC
GT142	60.67	9.41	4.1	87.32	33.98	12.3
GT188	16.21	13.42	5.4	84.17	31.66	9.5

%M1: Percent of events having fluorescence >99% of unstained cells.

GMCF: Geometric mean channel fluorescence of all organisms detected in the sample.

FIOC: Fold-increase over control, calculated by dividing the experimental geometric mean fluorescence by the geometric mean fluorescence of the unstained controls, which corrects the fluorescence readings for yeast autofluorescence.

Table 2. Effect of incubation of *Candida* in serum at 37°C on CD11b/Mac-1 staining.

Strain and condition	Incubation with 10% fetal calf serum			Incubation with 10% human serum		
	%M1	GMCF	FIOC	%M1	GMCF	FIOC
GT142 with serum	70.51	14.03	5.0	5.24	21.51	6.1
GT142 without serum	87.32	33.98	12.3	87.32	33.98	12.3
GT188 with serum	33.21	12.58	4.3	2.51	27.29	3.6
GT188 without serum	84.17	31.66	9.5	84.17	31.66	9.5

For abbreviations, see Table 1.

Table 3. Effect of estrogen on binding of CD11b/Mac-1 antibody to *Candida* during culture in YNB (yeast nitrogen broth) with 10% fetal calf serum for 2 hr or 18 hr.

Strain and condition	Incubation for 2 hr			Incubation for 18 hr		
	%M1	GMCF	FIOC	%M1	GMCF	FIOC
GT142 methanol control <sup>a</sup>	75.70	21.45	5.5	74.69	17.58	5.4
GT142 estrogen 1x10 <sup>-9</sup> M	77.81	16.89	5.5	76.05	19.12	5.8
GT188 methanol control <sup>a</sup>	78.92	18.95	6.3	68.33	17.03	5.2
GT188 estrogen 1x10 <sup>-9</sup> M	54.21	20.18	6.4	69.08	19.14	5.4

For abbreviations, see Table 1

<sup>a</sup>Estradiol-17 $\beta$  was dissolved in methanol for use in these experiments. Control cultures contained methanol in place of estrogen in methanol. The final concentration of methanol in the control and experimental cultures was 1% v:v.

Table 4. Effect of growth temperature and serum on concanavalin A binding to *Candida* during culture in YNB for 18 hr.

Strain	25°C without fetal calf serum			37°C without fetal calf serum			37°C with 10% fetal calf serum		
	%M1	GMCF	FIOC <sup>a</sup>	%M1	GMCF	FIOC <sup>a</sup>	%M1	GMCF	FIOC <sup>a</sup>
GT142	99.90	5365.21	516.4	100.0	4817.92	569.5	99.70	5298.55	745.2
GT188	100.0	4446.27	360.9	100.0	5293.97	416.2	99.87	5640.43	507.7

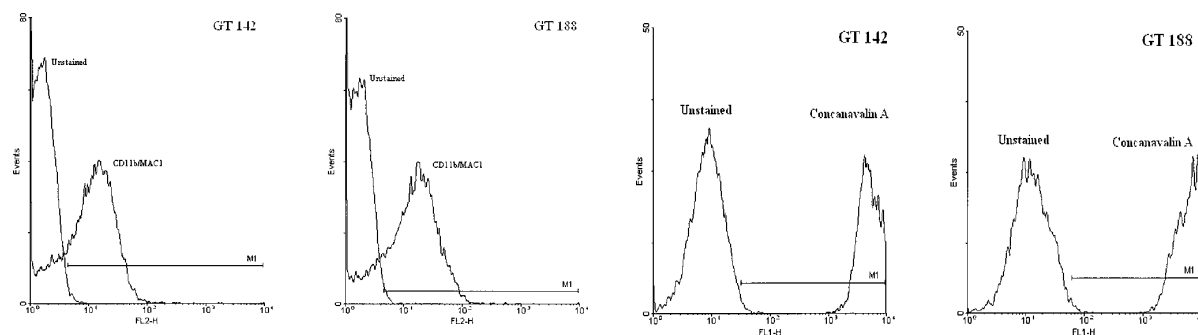


Fig. 1. Phycoerythrin-labeled CD11b/Mac-1 antibody staining of 2 strains of *Candida albicans*. Histogram plots the number of yeast cells (events) on the vertical axis against relative fluorescence of these cells. The unstained cells have minimal fluorescence (autofluorescence) and form a population to the left. Marker M1 excludes 99% of the unstained cells and shows that most stained cells have fluorescence above background.

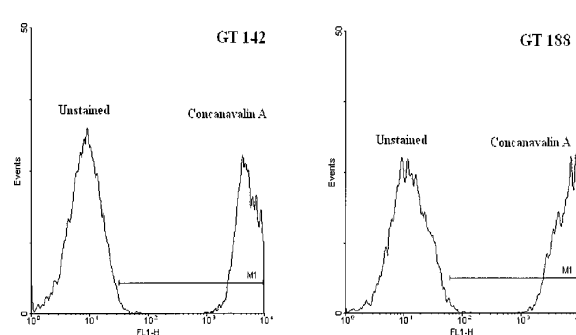


Fig. 3. Concanavalin A staining of 2 strains of yeast. Unstained control yeast showed autofluorescence with a peak around FL1-H = 10 and stained cells showed 7100 times more fluorescence than controls. Virtually all stained yeast (%M1 = 100%) showed strong FL1-H signal with GMCF at 4818 for strain GT142 and 5294 for strain GT188.

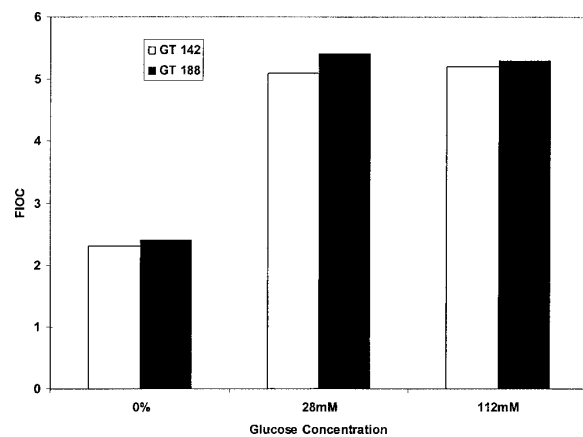


Fig. 2. CD11b/Mac1 staining of yeast grown in different glucose concentrations. CD11b/Mac-1 antibody binding is indicated by fold increase in the geometric mean fluorescence of the total cell population, which correlates with the glucose concentration. Yeast grown in a range of glucose concentrations were either unstained (to establish background levels of fluorescence) or stained with CD11b/Mac1. For each glucose concentration the fold-increase over control was calculated by dividing the fluorescence of the unstained (autofluorescence) control cells by that of the stained cells.

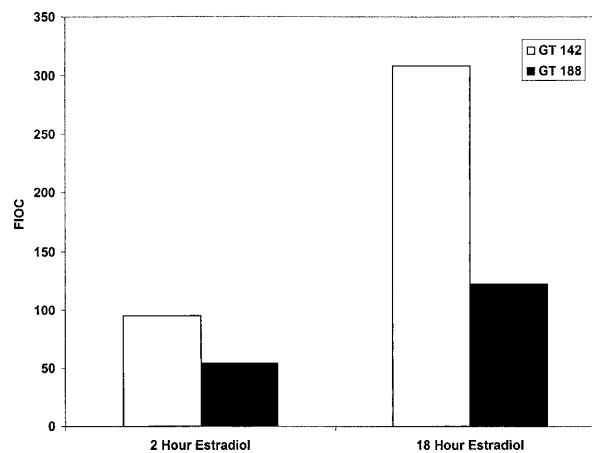


Fig. 4. The effect of growth in  $1 \times 10^{-9}$  M estradiol 17 $\beta$  on concanavalin A staining of yeast. FIOC (fold-increase over control) is calculated by dividing the GMCF of the estrogen treated organism by the GMCF of the estrogen-free control. Strain GT142, which shows increased growth in the presence of estradiol, also shows greater sensitivity to estrogen with respect to concanavalin A binding, compared to strain GT188, which does not show growth stimulation in the presence of estradiol.

broth medium at 2 different temperatures. The effect of growth temperature on binding of CD11b/Mac-1 was minimal, with the elevated temperature resulting in a slightly greater expression of CD11b/Mac-1 binding capacity (Table 1) than occurred at 25°C. Based on this finding, subsequent experiments were carried out at 37°C.

Growth of two yeast strains in YNB with 28 mM glucose, with or without 10% fetal calf serum, at 37°C, showed that serum decreased the binding of CD11b/Mac-1 for both test organisms, although the magnitude of this effect was small. The serum-grown yeast consistently exhibited a slight decrease in antibody binding over the non-serum grown yeast based on both GMCF and %M1 parameters. Yeast grown in human serum showed a high degree of germination as confirmed by microscopic examination, but iC3b receptor analog expression in both yeast strains (Table 2) did not appear from flow cytometry data to be consistently affected. Notably, *Candida* harvested from human serum-supplemented cultures had very large masses of mycelial fungi that were too large to be sampled by the flow cytometer. Therefore samples analyzed by flow cytometry were most likely reflective only of yeast forms.

Prior studies [7] of germ tube formation indicated that estradiol is an important factor influencing germination. A study of the potential of estradiol to affect CD11b/Mac-1 binding was performed by incubating *Candida* strains in YNB with  $1 \times 10^{-9}$  M estradiol-17 $\beta$  for 2 or 18 hr at 37°C. The results (Table 3) show that for both strains of *Candida* under all conditions tested, the effect of estrogen was equivocal, despite the fact that the effect of estrogen on growth rates of these 2 test organisms differed markedly. Because these studies with CD11b/Mac-1 antibody staining indicated little effect of environmental factors on binding of this antibody, the studies on temperature, serum, and estrogen were repeated to confirm these observations. The repeated studies gave results similar to our initial observations.

**Surface mannoprotein.** Although the specific surface marker detected by CD11b/Mac-1 staining proved minimally affected by anything except glucose

concentration, the non-specific putative virulence factor, mannosylated proteins on the yeast surface, may be regulated by environmental factors. Concanavalin A is able to bind mannose proteins on the yeast surface, allowing the use of FITC-labelled concanavalin A detected by flow cytometry as a probe for yeast mannosylation. Fig. 3 confirms that both strains of yeast bind fluorescinated concanavalin A, indicating substantial surface display of mannosylated proteins. The specificity of concanavalin A was indicated by decreased staining when 28 mM mannose was added to the cell suspension prior to addition of the labeled concanavalin A (data not shown). Glucose also reduced concanavalin A binding, but much less effectively than mannose did. In parallel with CD11b/Mac-1 studies, environmental growth conditions of temperature or serum altered concanavalin A binding to yeast. Table 4 indicates that growth at 37°C produced relatively more concanavalin A reactivity than growth at 25°C, and that growth in serum (10% fetal bovine serum) enhanced concanavalin A binding.

To confirm that growth conditions influenced concanavalin A binding, GT142 and GT188 *Candida albicans* were incubated with  $1.0 \times 10^{-9}$  estradiol overnight at 37°C. Fig. 4 shows that estradiol effectively increased the concanavalin A binding to both of these yeast strains. This was notable since the organisms behaved differently in regard to growth yield in the presence of estradiol.

**Phagocytosis.** The relationship of mannose-reactive protein binding to virulence is unclear. If the organism increases its expression of mannose proteins, mannose binding lectin in serum could bind in greater abundance, resulting in enhanced opsonization of the organism. Thus, an attempt was made to address the functional implication of these findings by determining how effectively neutrophils phagocytosed *Candida* cultivated with and without estradiol. This functional analysis employed human neutrophils, reacted with FITC-labeled yeast. Acquisition of fluorescence by neutrophils indicated binding of yeast to the neutrophils, although it did not confirm the yeast were internalized. A relatively new technique, developed by Busetto et al [14], uses trypan blue

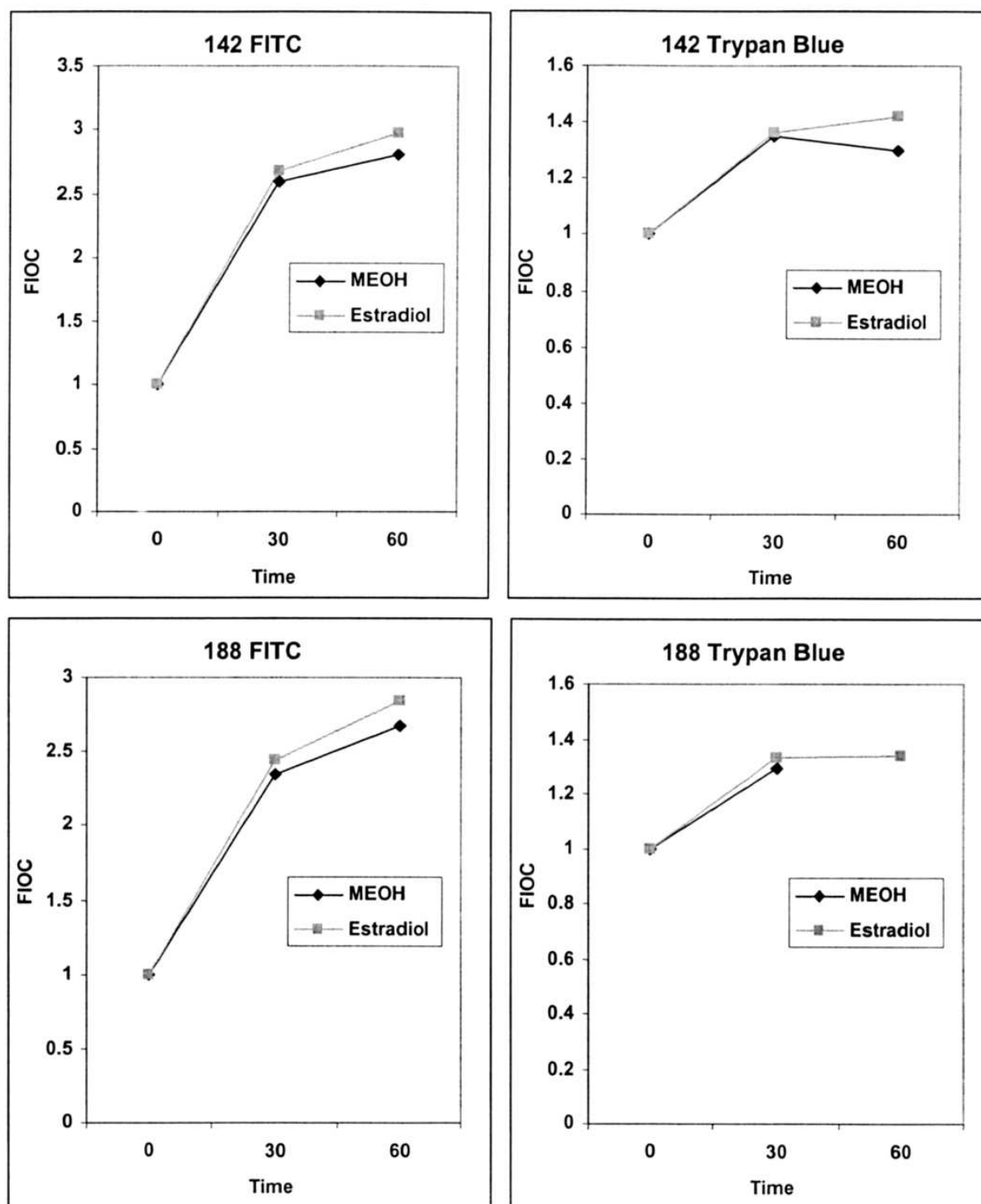


Fig. 5. Phagocytic efficiency of PMN toward yeast grown in estradiol-17 $\beta$  or control (containing methanol in place of estradiol-17 $\beta$ ). FIOC was calculated by dividing the GMCF of the experimental tube by the GMCF of the unstained control. FITC stained yeast indicate yeast associated with phagocytic cells; trypan blue stained yeast indicate yeast that have been internalized.

staining of yeast to distinguish intracellular from extracellularly bound organisms. Trypan blue provides an FL2-H signal when yeast have been internalized by phagocytic cells since internalization alters the fluorescence emission spectrum of trypan blue stained yeast.

Data for this experiment are presented in Fig. 5. As expected, yeast were opsonized by exposure to pooled normal human serum. The trypan blue signal, which parallels the FITC signal, indicated that the yeast were internalized. Interestingly, the yeast grown in the presence of estradiol were no more resistant to phagocytosis than yeast grown without estradiol. In fact, yeast grown in estradiol tended to be slightly more susceptible to phagocytosis.

### Discussion

As investigators have become better acquainted with the virulence attributes of microorganisms that can associate commensally with the host as normal flora, they have also recognized that virulence occasionally expressed by these microbes appears to be a consequence of multiple factors working in concert [17]. Investigation of *Candida albicans* and its virulence factors provides an interesting study in host-parasite interaction because, despite being the most commonly encountered fungus in clinical infections, this organism most frequently associates stably with mucosa without causing symptoms. Previous research [6-10] indicated that certain virulence-related characteristics of *Candida* could be regulated by conditions within its environment. A particular focus on the role of mammalian hormones as environmental cues that alter expression of virulence-related factors has emanated from the recognized propensity of colonized women to develop symptomatic candidiasis as pregnancy progresses [18]. Prior studies have primarily been concerned with cytoplasmic or intracellular factors influenced by estrogen and other growth conditions [6-10]. The present report extends these studies to the factors associated with the fungal surface. Since the surface of mucosal pathogens is important in attachment to the affected tissue, studies of the fungal surface may have implications for human mucocutaneous candidiasis.

Microorganisms that infect mucosal sites are known to have specific and non-specific mechanisms that allow them to adhere to the host tissue. Cell surface hydrophobicity is among the nonspecific microbial factors that promote tissue adherence [11]. Previous work from our laboratory has determined that yeast surface hydrophobicity may be influenced by environmental conditions such as temperature, the concentration of proteins, and carbohydrate levels. Whereas growth of the fungal organisms in estradiol affects growth rate [6], expression of HSP90 [9], germination [7], and other phenotypic features [8,10], estrogen did not appear to alter hydrophobicity [17], nor was expression of the iC3b receptor analog upregulated by estradiol in the present study. While additional yeast strains could have been tested in this experiment, the fact that 2 organisms with distinct growth reactions to estradiol yielded negative results suggested that testing of additional strains would not necessarily be useful.

The detection of iC3b receptor analog on fungal cells was first reported by Hostetter's group (13,16) using fluorescent-labeled antibody measured by flow cytometry. Although a similar method was used in this study, the same antibody as that used by Hostetter et al [16] was not available for this research. A commercial CD11b/Mac1 antibody which was expected to bind to the same ligand as Hostetter's antibody proved to be a valid alternative of Mol-1. While it was impossible to know if the reagent we used was bound to precisely the same antigen as Hostetter's, significant binding of CD11b/Mac-1 to the yeast and upregulation with increased glucose concentration was consistent with the behavior of iC3b receptor analog. This factor is of interest as it may possibly decrease phagocytosis of yeast and has therefore been suggested to subvert host defense. An unexpected finding was that human serum failed to increase CD11b/Mac-1 binding of yeast since mycelial forms are expected to have high levels of iC3b receptor analog [16]. However, *Candida* grown overnight in human serum formed very large mycelial clumps, and these appeared to be too large to be sampled by the flow cytometer. This may explain why flow cytometry failed to detect the iC3b receptor analog expression by mycelia. Further study of yeast exposed very briefly to serum (before massive

mycelial transformation occurs) might show early signs of iC3b receptor analog upregulation and will be addressed in future studies.

Application of flow cytometry to probe the microbial surface has been used to a lesser degree than it has for characterizing mammalian cells in immunology and cancer research. However, flow cytometry has the ability to interrogate the surfaces of large numbers of microorganisms at a high rate of speed. This means that relatively small differences in the fluorescence parameters in the range of 3-5% within an individual controlled experiment are meaningful since most experiments examine 10,000 yeast cells. In the present study, when differences between experimental cultures and their controls were small, the experiment was repeated to confirm the lack of effect. This was the case for the effect of serum and estradiol on CD11b/Mac-1 binding. While absolute fluorescent parameters such as %M1 and GMCF varied considerably between repeated experiments, measures that accounted for fluorescence background and experimental controls (fold-increase over control) typically varied less than 10% between repeated experiments, which implies that differences <10% between individual experiments are not significant. Despite its advantages, flow cytometry of fungi should be extended by microscopy with immunofluorescent detection of surface iC3b receptor analog, especially when substantial levels of mycelial transformation are present.

Because concanavalin A binds to mannose and glucose residues, it is used as a surrogate marker for the mannoproteins or mannans that are considered to be contributors to surface hydrophobicity and tissue binding. Since mannose, more than glucose treatment, reduced the amount of concanavalin A binding, it appeared that this reagent served primarily as a marker for mannosylated surface markers, and to a lesser extent for glycosylated proteins. Concanavalin A binding was also inhibited by addition of serum to the incubation medium in the present study. Because mannoproteins may provide binding sites for serum factors such as collectin, a mannose binding lectin (MBL) that can act as an opsonin, the level of surface mannose expression could have implications for phagocytosis of yeast [19].

Mannose proteins and iC3b have each been reported to influence the ability of neutrophils to phagocytize yeast through different mechanisms. Consequently we wished to know if different environmental conditions affected these 2 factors. Estrogen, which previously was shown to affect a variety of putative intracellular virulence factors, did not appear to alter expression of iC3b receptor analog, but it did significantly increase yeast surface mannose protein expression. Based on the criterion that FIOC>10% probably reflects true differences in reagent binding, we were only able to confirm the estrogen effect on concanavalin A binding and not on CD11b/Mac-1 binding.

A preliminary investigation of the phagocytic efficiency of human neutrophils toward yeast grown with or without estrogen indicated that estrogen slightly increased the phagocytosis (yeast attachment to neutrophils as well as yeast internalization) despite the facts that it did not increase iC3b receptor analog, but did clearly increase expression of mannose proteins. While phagocytosis of estrogen-grown yeast was not significantly affected, the data in Fig. 5 suggest a trend toward increased phagocytosis among estrogen-cultured cells and probably deserve evaluation in future research.

As noted in clinical practice, propensity of pregnant women to develop symptomatic yeast vaginitis may be due not only to the way in which elevated estrogen levels affect known virulence factors, but may also be influenced by estrogen effects on tissue binding and phagocytosis. The present study suggested that direct estrogenic effects on yeast phagocytosis relate to mannose proteins and may tend to enhance phagocytosis only slightly.

The present study shows that flow cytometry is a useful tool for evaluating the expression of surface properties of microbial pathogens, providing sufficient discrimination between microbial features regulated by environmental cues. In this regard, the iC3b receptor analog appears to be stable under a variety of conditions. However, serum and estradiol affected test organisms with respect to mannose protein expression. The effect of estrogen on these organisms may vary with the intrinsic growth response to estrogen. No significant effect on phagocytosis was demonstrated in this study,

although the preliminary nature of the phagocytosis experiments suggest that further evaluations are warranted.

While it is tempting to envision that advances in antimicrobial drugs may obviate the need for complete understanding of microbial virulence, the discovery of agents that directly interfere with virulence factors offers an interesting approach to control infectious agents and could give rise to new types of antimicrobials. As we continue to elucidate fungal factors that are regulated by mammalian hormones, we may discover mechanisms by which host defenses are affected and new methods of inhibiting microbial factors that affect host defenses.

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