Pathogenicity of *Candida albicans*, the most virulent *Candida* species, is attributed to a diverse array of virulence factors, including the abilities to germinate and to adhere to host epithelial cells. Frequently, portals of entry for *C. albicans* are mucous membranes protected by secretory immunoglobulin A (SIgA). However, the role of SIgA in local host defense against *C. albicans* is currently unclear. This study’s objectives were to investigate effects of SIgA upon *C. albicans*’ germination and adherence to HEp-2 cells, using a germination assay with phase contrast microscopy and flow cytometry, respectively. Eighty percent (8/10) of *C. albicans* strains exposed to a 3-fold dilution series of pooled human SIgA, ranging from 1667 to 62 µg/ml, experienced a decrease in germination, whereas two showed either no change or an increase. Removal of *Candida*-specific SIgA negated the reduction in germination. SIgA did not affect germ tube length. To investigate the influence of SIgA upon adherence of *C. albicans*, eight strains were exposed to 10-fold dilutions of SIgA, ranging from 500 to 0.5 µg/ml, fluorescently stained, and incubated with HEp-2 cells prior to analysis. Two of eight strains exhibited increased adherence after SIgA exposure. In contrast, six strains demonstrated no significant change in adherence. In conclusion, germ tube formation is inhibited by *Candida*-specific SIgA. However, adherence of *C. albicans* is largely unaffected by SIgA. These findings suggest SIgA has a novel role of controlling germination and subsequent invasion of the host by *C. albicans.*
INFLUENCE OF SECRETORY IMMUNOGLOBULIN A UPON GERMINATION FREQUENCY AND ADHESION OF CANDIDA ALBICANS

A THESIS

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In partial fulfillment of the requirements for the degree of
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Department of Microbiology

by
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2003

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INTRODUCTION

Taxonomically, Candida is a heterogenous, anamorphic genus belonging to the Saccharomycetales in Kingdom Fungi Phylum Deuteromycota Class Blastomycetes (21). This genus is comprised of approximately 200 species, including C. albicans, which is the etiological agent of up to 75% of candidal mycoses (28) and is the most virulent species of Candida (14, 43). C. albicans was first described by Lagenbeck, in 1839, as a parasite of humans infected with aphthae, as thrush or oral candidiasis, was previously known (30). The classification of the etiologic agent of thrush remained controversial until 1923, when Berkhout assigned its current genus (36).

C. albicans is a component of the normal flora in the alimentary tract and on mucocutaneous membranes (3, 14, 28, 30, 37, 43) of up to 65% of healthy, immunocompetent human hosts (20). Although this fungus is a commensal, it possesses virulence factors, which enable it to become an opportunistic pathogen when disruption of either the normal flora or the immune system occurs (8, 14, 29, 39, 43). For example, up to 90% of human immunodeficiency virus (HIV)-seropositive patients suffer from candidal mycoses at some point in the viral infection (15).

Candidiasis

Candidiasis occurs worldwide. It was first described in approximately 400 B.C. by Hippocrates as oral thrush in humans, but the responsible organism was not identified until 1839 (30). In the late 1700s, von Rosenstein and Underwood documented that oral candidiasis primarily affected infants (30). Today, candidiasis, characterized by widely variable, i.e., acute or chronic, superficial or disseminated, clinical signs is an opportunistic mycosis of the increasingly prevalent immunocompromised population (14, 29, 30).
Classification of candidiasis is based on either the degree of invasiveness, i.e., superficial, invasive, or disseminated candidiasis, or the anatomic site, i.e., oral, gastrointestinal, cutaneous, or mucocutaneous candidiasis. In oral candidiasis, hyphae, which are the ca. 3 µ diameter filamentous form, and blastoconidia, which are ca. 3-5 µ oval-to-elliptical morphologic forms, respectively, of C. albicans form a thick pseudomembrane over the oral tissues and can be extensive enough to interfere with swallowing and breathing (14). If left untreated, oral candidiasis may disseminate as blastoconidia, via fungemia, subsequently leading to life-threatening fungal colonization of vital organs including the heart valves and liver (14, 30, 43).

Treatment of candidiasis is limited to a small number of antimicrobial chemical classes, primarily polyenes, triazoles, and echinocandins, which alter cell membrane ergosterol, interfere with ergosterol synthesis, and inhibit cell wall β-glucan polymers, respectively (38, 46). Polyene antifungals, such as oral topical nystatin and systemic amphotericin B, are commonly used to treat thrush and invasive candidiasis, respectively (12, 17, 38). However, amphotericin B is nephrotoxic and therefore, reserved for critical cases. In recent years, antifungal drug-resistant C. albicans strains have developed (3, 41, 46). Due to the harsh host toxicity of these drugs and the emergence of resistance, there is a great need for an effective prophylaxis against C. albicans.

**Virulence factors of C. albicans**

C. albicans exhibits a diverse array of virulence factors that are absent or negligible in other Candida species. These virulence factors contribute to the versatility of C. albicans, allowing it not only to invade the host via a wide variety of entrance portals, but also to demand that the candidiasis-resistant host exhibit competent and multifaceted immune responses.
Additionally, it is important to note that these virulence factors are expressed neither continually nor simultaneously, but are exhibited in a dynamic, rapidly fluctuating manner.

*C. albicans* is adept at expressing a variety of virulence traits, including phenotypic switching, hypha formation, thigmotropism, displaying cell surface hydrophobicity and surface virulence molecules, molecular mimicry, production of lytic enzymes, rapid growth rate, and having undemanding nutrient requirements (39). Rapid phenotypic switching among displayed cell surface molecules and morphologic forms is critical for the pathogenesis of *C. albicans*. This ability allows *C. albicans* to qualitatively and quantitatively adapt expression of its virulence factors to different host microenvironments (39), creating a diverse population, which increases the likelihood of successful host invasion and immune system evasion. Since *C. albicans* lacks the capacity for meiosis and “has no other remarkable phenotypic characters” (39), rapid phenotypic switching confers a selective advantage allowing the opportunistic fungus to overcome the host. For example, *C. albicans* rapidly displays surface virulence molecules, particularly a mannoprotein adhesin (11, 23), which enhance binding to host tissue, a necessary step for successful colonization (39, 49). In addition to surface virulence molecules, which confer specific attachment mechanisms, *C. albicans* uses non-specific mechanisms exemplified by its attachment-enhancing cell surface hydrophobicity. Furthermore, *C. albicans* secretes broad-spectrum lytic enzymes, such as proteinases, phospholipases, and lipases, which facilitate invasion of host tissue.

As an adjunct to its invasive mechanisms, *C. albicans* uses complementary immunological evasive mechanisms to make itself less recognizable to the host. These mechanisms include molecular mimicry, a relatively fast growth rate that can overwhelm the
immune system, and undemanding nutrient requirements, which allow the fungus to survive in varied environments (39).

Among *C. albicans*’ array of virulence factors, dimorphic transition between yeasts and hyphae is fundamental. Both *in vitro* and *in vivo*, *C. albicans* can undergo three distinct morphogenetic processes, i.e., blastoconidium (yeast formation), pseudohyphal formation, and hyphal formation (30). Both pseudohyphal and hyphal formations begin as cylindrical outgrowths from a blastoconidium. However, during pseudohypha formation, a septum is formed between the blastospore and the cylindrical outgrowth, whereas in hypha formation the initial stage is a “germ tube” that lacks the septum between the daughter cells and the mother cell (30). The germ tube is distinguished by parallel walls that do not constrict at the point of attachment to the parent cell (14, 30, 35, 43). Morphological switching can be induced by several environmental factors, including pH, temperature, and nutritional conditions (2, 17, 33). During the transition from commensal to opportunistic pathogen, contact between *C. albicans*’ germ tube and the host tissue is facilitated by the germ tube’s cell surface hydrophobicity at 37°C and adhesion (8, 10, 13, 22, 23, 24, 25, 26, 36, 39, 44). The hyphae tightly adhere to the host epithelium and orchestrate thigmotropism, or contact sensing. Subsequently, the hyphae implant into the host epithelium, branch longitudinally, and produce yeast forms that are readily able to disseminate, colonize, and invade vital organs, leading to severe morbidity and even mortality (39, 51).

**Secretory IgA**

Secretory immunoglobulin A (SIgA) is composed of two serum IgA monomers joined by a J chain. The conversion to SIgA begins when dimeric IgA, which comprises only 15-20% of total serum antibodies (1), binds to the polymeric immunoglobulin receptor (pIgR) on the
basolateral surface of mucosal epithelial cells and is actively transported through the cells to the apical membrane. Once the apical membrane is reached, the external part of the pIgR is cleaved, leaving part of the receptor covalently joined to the IgA dimer. The bound receptor fragment, known as the secretory piece, differentiates SIgA from serum IgA (50).

Since the most common portal of entry for *C. albicans* is mucous membranes, which are protected by secretions, such as saliva, tears, gastrointestinal fluid, breast milk, and colostrum, that contain protective secretory immunoglobulins A (SIgA) and M (SIgM), these antibodies are the first line of humoral defense that *C. albicans* must overcome to successfully colonize a host. For example, SIgA, the predominant secretory antibody, is associated with protection of mucous membranes against invasion by bacteria and protozoa by binding to the organisms and limiting adhesion (31, 34, 50). Bound SIgA decreases bacterial hydrophobicity due to a glycosylated Fc region and the hydrophilic secretory piece. Furthermore, stereochemical interactions of SIgA bound to bacteria block the ability of bacterial adhesins to bind to the complementary host receptors (34, 50). SIgA binds to the protozoan, *Giardia lamblia* (31), and binds to and agglutinates bacterial cells, but it kills neither protozoa nor bacteria nor does it enhance phagocytosis (50). Although it is not generally considered a complement mediator, it can trigger the alternative complement pathway (40, 50, 52) and also acts to degranulate IL-3-primed basophils (27). Thus, there is significant information on the anti-bacterial and anti/protozoal activity of SIgA (16, 19, 31, 45, 54). However, SIgA’s role in humoral defense against fungi, such as *C. albicans*, is largely unexplored.
Specific Aims

Because little is known about the influence of SIgA upon C. albicans, the goal of this study was to determine the effects of SIgA on C. albicans' ability to form germ tubes and to adhere to human epithelial cells. The following specific aims were designated:

1) To assess the frequency of germ tube formation and the length of germ tubes in C. albicans post-exposure to SIgA compared to non-exposed controls. The following hypothesis was investigated: Does SIgA exposure result in statistically significant reductions in both the frequency of germ tube formation and the length of germ tubes? Methods used by other investigators, including in vitro cultural induction of germination (30, 44) and visualization via phase contrast microscopy (44), were chosen to provide data that were evaluated via two-way statistical analysis of variance (ANOVA).

2) To evaluate the comparative binding of C. albicans pre-exposed to SIgA and non-exposed C. albicans controls to SIgA to human buccal epithelial cells. The following hypothesis was examined: Does SIgA exposure result in a statistically significant reduction in adherence of C. albicans to buccal epithelial cells? Various methods have been used to evaluate binding of C. albicans to human buccal epithelial cells, including flow cytometry (11) and microscopy (9, 22, 48). A flow cytometric assay utilizing the fluorescent stain FUN-1® (36), was applied to evaluate adherence. This method was chosen because of its advantages, such as its ability to assess large numbers of cells in a relatively short time, to reduce experimental bias in interpreting results, and its efficiency compared to microscopy.
MATERIALS AND METHODS

Sources and maintenance of C. albicans strains  Nine C. albicans isolates, obtained from Shriners Hospital (Cincinnati, OH), in addition to two reference strains, C. albicans 90028 and C. albicans 28367 (American Type Culture Collection, Manassas, VA), were preserved for long-term maintenance in nutrient broth with 2.5% glycerol at –70°C. All cultures were incubated in the dark. The identity of each experimental C. albicans strain was confirmed using the germ tube test (32) and the Bacticard™ Candida test kit (Remel, Lenexa, KS). For the germ tube test, each strain was passed twice on Sabouraud dextrose agar (SDA) at 30°C to ensure viability. Next, the organisms were lightly inoculated into 10% peptone broth, to ca. 0.5 McFarland standard, and incubated in a 37°C water bath for 3 hours. Microscopic detection of germ tubes confirmed each C. albicans strain. Since up to 5% of C. albicans clinical isolates fail to produce germ tubes in vitro, and pseudohyphae of non-albicans Candida species may be mistaken for germ tubes in the clinical laboratory, the Bacticard™ Candida test kit was used to additionally confirm each strain’s identity by detection of the enzymes L-proline aminopeptidase and β-galactosaminidase.

Antibodies  Human secretory IgA (SIgA), serum IgA, and IgG, in lyophilized, pooled form, were purchased from ICN Pharmaceuticals (Cleveland, OH). An agglutination assay was used to determine the titer of Candida-specific antibodies in the pooled SIgA. Briefly, the C. albicans was grown on SDA for 24 hours at 37°C. The cells were diluted to 1.0 x 10^7 cells/ml in 0.05 M phosphate buffered saline (PBS), pH 7.2. To each well in a 96-well plate (Nunc, Rochester, NY), 0.05 ml of 0.85% saline was added. Fifty µl of rehydrated SIgA (500µg/ml) was added to the first well in each row. A two-fold dilution series was used to dilute the SIgA to the appropriate concentrations in subsequent wells. Next, 50 µl of C. albicans was added to each
well. The plate was incubated at 25°C for 24 hours and then each well was viewed for agglutination, signified by a diffuse layer of \( C.\ albicans \) on the bottom of the well, and confirmed microscopically. The final well in each row, containing only saline and \( C.\ albicans \), served as a negative control.

**Protein Adsorption** Candida-specific antibodies were removed from the pooled human SIgA using an adsorption protocol. Lyophilized pooled, human SIgA was rehydrated according to the manufacturer’s instructions. A 150 µl aliquot was reserved for later protein quantification, and the remaining SIgA was incubated with \( 1 \times 10^8 \) cells of \( C.\ albicans \) ATCC 28367 per ml for 1 hour at 4°C on a rocker. The suspension was then centrifuged for 10 minutes at 4000 rpm and the supernatant was removed. The protein concentration present in each of the reserved portions of SIgA supernatant was quantified using the Coomassie® Plus Protein Assay Reaction Kit (Pierce Biotechnology, Rockford, IL). Adsorption was repeated until the protein concentration remained constant for three consecutive quantifications. An agglutination assay with a titer of 0 using the adsorbed antibody, confirmed the removal of Candida-specific SIgA from the pooled SIgA.

**Germination Assay**

\( C.\ albicans \) cells, harvested from individual colonies of a 24 hour, 35°C, SDA plate, were suspended in 2 ml of phosphate buffered saline (PBS) pH 7.4 to a concentration of \( 6.5 \times 10^6 \) cells/ml. Germination was induced by adding 100 µl of 1% Bacto-peptone (Fisher Scientific Co., Pittsburgh, PA) to 100 µl of \( C.\ albicans \) suspension. Subsequently, 100 µl of the appropriate SIgA dilution, 62, 185, 555, 1667µg/ml, was added. The control substituted 50 µl of PBS for SIgA. The tubes were then incubated in a rotary shaker at 37°C, 150 rpm, in the dark for 90 minutes.
To determine whether SIgA inhibited *C. albicans’* reproduction, colony-forming units (CFU) were measured. One hundred microliters of SIgA-exposed *C. albicans* from the germination assay were diluted 1:200 in PBS. Twenty microliters of the diluted suspension were spread-plated onto SDA and incubated for 24 hours at 35°C, followed by viable counts using a Bantex Colony Counter (Fisher Scientific Co., Pittsburgh, PA).

After the removal of cells needed for calculation of CFU/ml, 98 µl of 95% ethanol and 2 µl of acid fuschin stain were added to fix and stain the *C. albicans* cells. Slides for microscopy were prepared by placing 10 µl of the suspension onto a clean slide, placing a 22 x 22 mm cover slip over the drop, and sealing the coverslip with nail polish. The slides were blinded by covering all labeling with opaque tape and randomly assigning a number to each slide. The suspensions were also streaked onto SDA and incubated overnight at 35°C to confirm the fixation of *C. albicans*. Three hundred sequentially encountered *C. albicans* cells were evaluated microscopically, following a specific path on the slide to prevent counting cells multiple times. Germ tubes of 100 germinated cells were also measured at 1000 x magnification, using an ocular micrometer with the ability to measure micron increments, to assess the effect of SIgA on the length of germ tubes.

**Adherence Assay**

*Preparation of C. albicans.* Seven clinical isolates of *C. albicans* obtained from Shriners Hospital (Cincinnati, OH) and ATCC 90028 were used in this assay. In preparation for the adhesion assay, all strains were grown on yeast extract peptone galactose (YPG) agar at 35°C in the dark for 24 hours. Two isolated colonies were chosen from the YPG agar and used to inoculate 100 ml of YPG broth. The cells were grown to late log phase by incubating at 30°C, 150 rpm, for 18 hours.
Preparation of HEp-2 cells. Briefly, HEp-2 19615 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s Modified Eagle’s Medium + 5% Serum Supreme, a synthetic fetal bovine serum (BioWhittaker, Walkersville, MD) supplemented with 10 µg/ml gentamicin sulfate and 100 µg/ml streptomycin sulfate in 100 x 15 mm tissue culture-coated Petri plates (Fisher Scientific, Pittsburgh, PA). The cells were cultured to confluence, washed once with PBS, pH 7.4, and scraped with a rubber policeman. The cells were vortexed to separate clusters of cells and suspended to a concentration of 4.0 x 10⁴ cells/ml (47).

Exposure and staining of C. albicans. Late-log phase C. albicans cells were washed twice with 20% glucose-10 mM Na HEPES solution (pH 7.2) and diluted to a concentration of 1.0 x 10⁵ cells/ml. Next, 900 µl aliquots of this cell suspension were exposed to 100µl of SIgA, diluted 10-fold from 500 to 0.5 µg/ml, for one hour on a shaker at 37°C, 150 rpm. Also, as positive and negative controls, respectively, C. albicans was exposed to either human serum IgA or human serum IgG that had been diluted to 500 µg/ml.

C. albicans was stained with FUN-1® (Molecular Probes, Inc, Eugene, Oregon), a two color fluorescent probe used to detect yeast viability, and calcofluor white (Sigma, St. Louis, MO), a fluorescent fungal wall-labeling reagent. The FUN-1® stock was diluted to 5 mM using 20% glucose-10 mM Na HEPES solution (pH 7.2). One µl of 5 mM FUN-1® and 1 µl of 25 mM calcofluor white was added to each aliquot of C. albicans to be stained. The aliquots were incubated for 30 minutes at 37°C in the dark.

Adhesion Assay. One ml of HEp-2 cellular suspension was combined with 0.5 ml of antibody-exposed C. albicans that had been previously stained with FUN-1® and calcofluor white stain in 12 x 75 mm polystyrene snap-cap tubes. These tubes were incubated on a rotary shaker at 37°C for 1 hour at 150 rpm and then analyzed, along with controls, using flow
cytometry. To investigate the importance of *Candida*-specific antibodies, the assay was repeated using *C. albicans* ATCC 28367 and pre-adsorbed human SIgA.

**Statistical analysis** SAS® statistical analyses were employed to determine the statistical significance of all data collected. Error bars displayed in the results section signify standard error for the experiment. This method was chosen due to the large number of observations that were made for each assay. For graphs using letters to show significance (Figures 1B, 2B, 4B, and 5) significance was determined using the Bonferroni correction method. This procedure corrects for the significance level based on the number of tests performed. For example, if the significance level was originally set at 0.05 and ten comparisons are being made, the corrected significance level is 0.005, or 0.05/10. Columns on a graph sharing a designated letter are not considered significantly different.
RESULTS

Agglutination  To determine the amount of *Candida*-specific antibody present in the SIgA, IgA and IgG, an agglutination assay was carried out, as described in Materials and Methods. This assay confirmed the presence of specific antibody directed towards each *C. albicans* strain analyzed in the germination and adherence assays. The strain-specific titer for the SIgA, IgA, and IgG varied (Table 1).

Inhibition of germination by SIgA  Inhibition of frequency of germ tube formation was detected using the germination assay described above. Briefly, germination of *C. albicans* was induced by incubation with peptone and three dilutions of pooled human SIgA. Cells were fixed with ethanol, stained, and visualized using phase contrast microscopy. Slides were then blinded, and the cells were visualized using phase contrast microscopy. Three hundred cells were counted and classified as either germinated or non-germinated.

A differential effect of SIgA dose upon frequency of germ tube formation in different *C. albicans* strains was demonstrated (Fig. 1A). For example, 80% (8/10) of tested *C. albicans* strains exhibited a significant reduction in germination frequency, ranging from 2% to 38%, post-exposure to 62, 185, 555, or 1667 µg SIgA/ml, whereas 20% either demonstrated no effect (Ca 900328) or a significant increase in germination (Ca 870097). Importantly, the average germination rate overall was significantly reduced by exposure to 555 µg and 1667 µg SIgA/ml (Fig. 1B). Two-way ANOVA revealed the effect of dose upon germination frequency is significant (p<0.0001, dF=4, F=20.06).
Table 1. Titer of SIgA, IgA, and IgG antibodies specific for each *C. albicans* strain used in the germination and adherence assays$^a$.

<table>
<thead>
<tr>
<th><em>C. albicans strain</em></th>
<th>Titer SIgA</th>
<th>Titer IgA</th>
<th>Titer IgG</th>
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<tbody>
<tr>
<td>Ca 28367</td>
<td>512</td>
<td>512</td>
<td>8</td>
</tr>
<tr>
<td>Ca 850383</td>
<td>256</td>
<td>512</td>
<td>4</td>
</tr>
<tr>
<td>Ca 870097</td>
<td>128</td>
<td>ND$^b$</td>
<td>ND$^b$</td>
</tr>
<tr>
<td>Ca 90028</td>
<td>128</td>
<td>64</td>
<td>8</td>
</tr>
<tr>
<td>CaSH 1</td>
<td>512</td>
<td>256</td>
<td>4</td>
</tr>
<tr>
<td>CaSH 2</td>
<td>512</td>
<td>ND$^b$</td>
<td>ND$^b$</td>
</tr>
<tr>
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<td>128</td>
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<td>CaSH 6</td>
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<td>16</td>
</tr>
<tr>
<td>CaSH 8</td>
<td>512</td>
<td>64</td>
<td>8</td>
</tr>
<tr>
<td>CaSH 9</td>
<td>512</td>
<td>256</td>
<td>16</td>
</tr>
</tbody>
</table>

$^a$ Agglutinating titer is defined as the last dilution displaying agglutination.

$^b$ Not determined (ND). Strains Ca 870097 and CaSH2 were not used for the adherence assay and, subsequently, they were not agglutinated using either IgA or IgG.
Fig. 1. Effect of dose of SIgA upon germ tube formation by *C. albicans*. (A) Germination frequencies (%) of individual *C. albicans* strains (n = 10 strains, comprised of 9 clinical isolates and 1 reference strain) exposed to increasing concentrations of pooled, human SIgA. (B) Effect of increasing SIgA dose upon mean germination frequency (%) of combined *C. albicans* strains (n = 10, the same isolates displayed in Fig. 1A). Bars, by dose, lacking a letter in common, are significantly different at the p<0.005 level of significance. For both Fig. 1A and 1B, results for three experiments are shown. Data was statistically analyzed using SAS® two-way ANOVA and least squares mean.
Statistical analysis demonstrated that strain variation had a large impact on the effect of SIgA upon germination. Figure 2 reveals the differential effect of strain in response to increasing doses of SIgA. The effect of strain upon germination frequency as a response to SIgA exposure was highly significant (p<0.0001, dF=9, F=58.19). Additionally, highly significant interaction between dose and strain was evident statistically, indicating that there is a differential effect of dose in different strains (p<0.0001, dF=36, F=3.75). For example, the frequency of germination in *C. albicans* strain 90028 was unaffected, regardless of the SIgA dose. However, CaSH 1 demonstrated reduced frequency of germination upon exposure to all doses of SIgA.

To investigate the possibility of non-specific interactions causing the observed results, the pooled human SIgA was pre-adsorbed with *C. albicans* ATCC 28367. *C. albicans* ATCC 28367 was chosen for this assay because it was a reference strain that showed a decrease in germination frequency upon SIgA exposure (Fig 3A), whereas the original reference strain *C. albicans* ATCC 90028 remained unaffected after SIgA exposure (Fig 1A). The germination assay was then repeated using the adsorbed SIgA. Removal of *Candida*-specific SIgA negated the inhibitory effects of SIgA (Fig. 3B), indicating that *Candida*-specific SIgA is necessary to reduce germination frequency.

**SIgA does significantly affect germ-tube length** Since SIgA is able to inhibit germination, the idea that it may also decrease germ tube length was investigated. Using the slides previously prepared to determine germination frequency, the germ tubes of 100 germinated cells were measured using an ocular micrometer with µ increments and phase contrast microscopy. The SIgA-induced decrease in *C. albicans*’ germ tube length was statistically relevant for the individual strains Ca 8950383, Ca 870097, Ca 90028, and CaSH 7. No significant effect was observed for strains CaSH 2, CaSH 4, CaSH 8, or CaSH 9 and strains
CaSH 1 and CaSH 6 demonstrated a statistically significant increase in germ tube length (Fig. 4A). The dose of SIgA significantly influences the length of formed germ tubes compared to the length of control germ tubes, which have not been exposed to SIgA (4B). Although the data suggest SIgA significantly impacts *C. albicans*’ germ tube length, it is probable that the reduction in germ tube length is small enough not to be biologically pertinent. This assumption is corroborated by the lack of a discernable trend in figure 4B. In addition, no morphologic aberrations, such as distorted germ tubes or blebbing of *C. albicans* cells, were observed.
Fig. 2. Variation in germ tube formation by *C. albicans* strains in response to increasing concentrations of SIgA. Germination frequencies (%) for each of 10 *C. albicans* strains (comprised of 9 clinical strains and 1 reference strain) exposed to five doses of SIgA are displayed. Results for three experiments are shown. Data were statistically analyzed using SAS® two-way ANOVA and least squares mean.
Graph showing the percentage germination of various *Candida albicans* strains in different concentrations of SigA. The x-axis represents different strains, and the y-axis shows the percentage germination.

Legend:
- □ 0 μg/ml SigA
- ● 62 μg/ml SigA
- △ 165 μg/ml SigA
- ◆ 555 μg/ml SigA
- ○ 1667 μg/ml SigA
Fig. 3. Effect of removal of *C. albicans*-specific antibodies from pooled SIgA upon germ tube formation. (A) *C. albicans* ATCC 28367 was employed because it is a reference strain shown to experience a reduction in germination upon exposure to SIgA. (B) Removal of *Candida*-specific SIgA negated the reduction in germination after exposure to SIgA. Pooled human SIgA was pre-adsorbed using Ca ATCC 28367. The experiment was conducted in triplicate and bars represent standard error. Removal of *C. albicans*-specific SIgA resulted in the restoration of germination ability in *C. albicans* ATCC 28367.
A.

![Graph showing percentage germination against [SlgA] (μg/ml)].

B.

![Bar graph showing percentage germination with different conditions: no SlgA, pooled SlgA, adsorbed SlgA].
Fig. 4. Effect of strain upon *C. albicans*’ germ tube length in response to increasing concentrations of SIgA. (A) Effect of strain upon *C. albicans*’ germ tube length in response to increasing doses of SIgA. Germ tube lengths for each of 10 *C. albicans* strains (comprised of 9 clinical strains and 1 reference strain) exposed to five doses of SIgA are displayed. (B) Mean effect of increasing SIgA dose upon germ tube lengths of combined *C. albicans* strains (n = 10, the same isolates displayed in Fig. 4A). Bars, by dose, lacking a letter in common, are significantly different at the p<0.005 level of significance. In both Fig. 4A and 4B results for three experiments are shown. Data were statistically analyzed using SAS® two-way ANOVA and least squares mean.
Effect of SIgA upon *C. albicans’* adherence is dependent upon strain  Adherence of SIgA-exposed *C. albicans* to HEp-2 cells was observed using a modification of the adherence assay published by Sethman et al. (47). Briefly, eight strains of *C. albicans*, seven clinical isolates and one ATCC reference strain, were incubated with 10-fold dilutions of SIgA and stained with the fluorescent dye, FUN-1®. The yeast cells were then incubated for 1 hour with HEp-2 cells. Flow cytometry was used to assess fluorescence of metabolically active yeast-HEp-2 cell complexes. For most strains (6/8), there was no significant increase in adherence of HEp-2 cells after exposure to 5 µg/ml of pooled human SIgA, and only 2 of the 8 total tested strains, CaSH 6 and CaSH 8, exhibited a dramatic increase in adherence after exposure to 500 µg/ml of pooled human SIgA (Fig. 5). Statistical analyses indicated that the effect of *C. albicans* strain upon adherence to HEp-2 cells following pre-exposure to pooled human SIgA was highly significant (p<.0001, dF=7, F=45.28). Furthermore, the effect of SIgA dose, and the strain-dose interaction were each highly significant, i.e., p<.0001, dF=4, F=32.91 and p<.0001, dF=28, F=5.66, respectively.

Figure 6 displays the impact of SIgA, IgA, and IgG upon adherence of *C. albicans* using 500 µg/ml of pooled human SIgA, IgA, and IgG, revealing that strain variation influences *C. albicans’* adherence to HEp-2 cells. In six of eight strains, exposure of *C. albicans* to pooled human SIgA and IgA caused a significant increase in adherence. In comparison, exposure to IgG resulted in little change for all strains.

To observe the impact of Candida-specific SIgA on adherence of metabolically active *C. albicans* to HEp-2 cells, pooled human SIgA was pre-adsorbed using *C. albicans* ATCC 28367, a strain demonstrating an increase in adherence upon exposure to pooled human SIgA. Interestingly, the results (Fig. 7) for the adsorbed and pooled SIgA were not significantly
different. Removal of *Candida*-specific SIgA did not result in a decrease in adherence as compared to pooled human SIgA, but displayed an increase in adherence compared to the control, which contained no antibody. Statistically, the fluctuation in response to SIgA can be explained by strain variation, difference in the response of each strain to dose, and the dose-strain interactions.
Fig. 5. Effect of SIgA dose upon adherence of *C. albicans* to HEp-2 cells. Eight strains of *C. albicans*, seven clinical isolates and one ATCC reference strain, were incubated with 10-fold dilutions of SIgA and stained with the fluorescent dye, FUN-1. The yeast cells were then incubated for 1 hour with HEp-2 cells. Flow cytometry was used to assess fluorescence of metabolically active yeast-HEp-2 cell complexes. The experiment was performed in triplicate and error bars represent standard error.
Fig. 6. Comparative effect of SIgA, IgA, and IgG on adherence of \textit{C. albicans} to HEp-2 cells. The adhesion assay previously described was repeated using 500 µg/ml of pooled human SIgA, IgA, and IgG. The assay also included \textit{C. albicans} that was incubated without any antibody. Results show that the effect of the antibodies varied among the strains of \textit{C. albicans}' assayed for adherence to HEp-2 cells. In general, exposure of \textit{C. albicans} to pooled human SIgA and IgA caused an increase in adherence. Exposure to IgG resulted in little change in adherence as compared to the control. Error bars represent the standard error.
Fig. 7. Comparative effect of adsorbed vs. pooled SIgA upon adherence of *C. albicans* ATCC 28367 to HEp-2 cells. To investigate the importance of *C. albicans*-specific SIgA on adherence of yeast cells to HEp-2 cells, *C. albicans* ATCC 28367, a strain previously demonstrating an increase adherence level upon exposure to SIgA, was used to pre-adsorb pooled human SIgA. The adherence assay was repeated in triplicate. Error bars represent the standard error.
DISCUSSION

*Candida albicans*, a major opportunistic fungal pathogen affecting the immunocompromised population, is the most pathogenic of the more than 200 *Candida* species. Its pathogenicity has been attributed to its large, multifaceted collection of virulence factors, including its ability to germinate and its ability to adhere to host epithelial cells. *C. albicans*, in the blastoconidial form, is often associated with commensalism and the hyphal, or germinated, form is associated with infection. Due to a dearth of knowledge regarding local immunity to *C. albicans*, the aim of this research was to investigate the impact of SIgA on two designated virulence factors, germination and adherence.

It is important to stress inherent difficulties that arise when working with SIgA and *C. albicans*. *C. albicans* is a diploid yeast, but has no known sexual cycle, which severely hinders mutagenesis, and thus, genetic analyses of the organism. During morphogenic transition, *C. albicans* isolates display complex strain dependence in gene expression. Because of the large number of genes required to express so many virulence factors, it is likely there will be virulence divergences between strains, as well as substantial alterations within a given strain (8, 39). Additionally, expression of cell surface antigenic molecules varies depending on several environmental factors, such as carbohydrate source, pH, temperature, and host niche. Antigenic components of the cell wall also differ between the yeast and the hyphal forms of *C. albicans*. Thus, the highly significant variation of strain and the dose-strain influence upon reduction of germination frequency in this current study are consistent with the literature (8, 34, 39).

The role of humoral immunity to fungal disease, specifically candidiasis, is poorly understood. Clearance of fungal infections is often associated with cellular immunity and the role of antibodies as a host defense mechanism is controversial. The antibody response to *C.
*C. albicans* is elaborate. Antibodies may be protective, non-protective, or infection enhancing, depending on the isotype and epitope to which they are directed. The complexity of antibody response and various effects of SIgA upon *C. albicans*’ germination explain why the cell-mediated immune system is the major host defense against fungal pathogens. Research has also demonstrated that agglutinating antibodies are not necessarily protective (18), making agglutination an unreliable measure of protective antibody within human serum.

This study focuses primarily on the impact of SIgA upon germination and adhesion of *C. albicans*, and contributes to a body of knowledge (18, 29, 34, 35, 38, 53) about the role of humoral immunity in first-line mucosal defense against *C. albicans*. Previous evidence supporting insignificance of SIgA in local humoral immunity against *C. albicans* includes findings that vaginal concentrations of anti-candidal SIgA were similar in women regardless of whether or not they have vaginal candidiasis, and, additionally, recurrent vaginal *C. albicans* infections were not prevented by specific anti-*C. albicans* IgA (4). Conflicting past evidence supports a significant role of SIgA in mucosal defense, demonstrating that passive immunity is conferred not only by vaginal fluid containing antibodies to mannan constituents and the aspartyl proteinase of *C. albicans* (7), but also by SIgA elicited by vaginal inoculation with a monoclonal antibody specific for yeast killer toxin, protected rats against candidiasis (42). Furthermore, work by Cassanova et al. (6) demonstrated that Fab fragments prepared from a monoclonal IgG antibody against a 260-kDa mycelium-specific *C. albicans* mannoprotein inhibited germ tube formation.

This study demonstrated that pooled, human SIgA, the key component of the host’s first-line defense, has the capacity to inhibit a key virulence factor of *C. albicans*, by inhibiting this opportunistic fungus’s germination frequency (Figures 1 and 2). This work expands upon
findings by Polonelli et al. (42), who demonstrated a fungicidal effect of SIgA upon C. albicans, by revealing that the humoral anti-candidal defense mechanisms are multifaceted. Importantly, the marked strain-to-strain variation of response to SIgA is highly consistent with the candidal attributes described in a classic review (8) of C. albicans putative virulence factors. C. albicans’ virulence is attributed to an array of virulence factors that are expressed in response to environmental signals, accounting for the variation observed between strains. In some regards, this current work contradicts evidence by Bohler et al., (4) indicating that SIgA levels do not correlate with protective levels against candidiasis. However, more recent evidence (5,6,7,8) supports a multifaceted role of antibodies against opportunistic fungi, providing scenarios for either invasion by C. albicans or defense against invasion. Dose of SIgA also plays a significant role in inhibition of germination. Importantly, the mean germination frequency of germination decreased as SIgA dose increased. The importance of Candida-specific SIgA as a germination inhibitor was corroborated by the restoration of germination when Candida-specific SIgA was removed by adsorption (Fig. 3).

Several mechanisms for the observed decrease in germination are possible. Candida-specific antibodies may be binding to surface molecules of the parent cells. Once the antibody binds, transduction signals involving transcription initiation may be blocked, thereby inhibiting the yeast-to-mycelium transition. Because germination of C. albicans may also be affected by cell density, it is also possible Candida-specific SIgA may be neutralizing quorum sensing molecules. This mechanism does not require binding to the yeast cell. By blocking the uptake of these quorum sensing molecules, SIgA inhibits the morphologic switch to the hyphal form.

Our study has also established that there is a statistically significant difference in germ tube length post-exposure to SIgA. However, there was no recognizable trend in the reduction or
increase in these specimens. This leads us to speculate that the reduction or increase in germ tube length is not biologically significant. Germ tube length was assessed using an ocular micrometer, with the capability of measuring length in µ, and phase contrast microscopy, leaving experimental error as an explanation for the observed response. Statistical analysis was conducted on ca. 15,000 observations, leading to an extremely small standard error, which is how the statistical significance was concluded. Biologic significance caused by such small differences in germ tube length is highly unlikely, compounded by the lack of an observable trend concerning the effect of SIgA upon germ tube length.

Thus, the data presented in this current study address a fundamental immunologic and mycologic debate regarding the contribution of antibody in natural defense against fungal infection, specifically candidiasis. Furthermore, since the investigated levels of SIgA included the natural range of SIgA in secretions, i.e., saliva, vaginal fluid, and breast milk, initially encountered by *C. albicans*, this *in vitro* study yields biologically relevant information with intriguing *in vivo* clinical implications. These results were not influenced by the titer of strain specific SIgA, as was expected, based on evidence of SIgA titer during bacterial and protozoal infections. This finding is supported by previous work (5, 18) showing that not all agglutinating antibodies are protective against candidiasis. A logical direction for future study includes the investigation of antibody specificity. Questions such as what is the target of the SIgA that inhibits germination in *C. albicans* and which epitope is targeted by infection-enhancing antibodies need to be answered to more fully understand *C. albicans’* response to SIgA.

Previous studies have documented that SIgA has differing effects upon adherence of *C. albicans* to epithelial cells. Vudhichamnong et al. (53) reported that SIgA directed towards specific epitopes on *C. albicans’* cell wall inhibits binding to epithelial cells. However, non-
specific SIgA enhances binding of the yeast to epithelial cells (53). In the current study, only 25% of the \textit{C. albicans} strains assayed showed a dramatic increase in adherence upon exposure to varying concentrations of non-specific SIgA. Six of the eight strains did not significantly respond to exposure to non-specific SIgA. Importantly, the two strains that had highly enhanced adherence when exposed to non-specific SIgA provide contradictory evidence to previously published research that only studied one strain of \textit{C. albicans} (53). The difference in adhesion results emphasizes the necessity of studying more than one strain of \textit{C. albicans}. Due to the variation between \textit{C. albicans} strains, several must be evaluated to understand an overall response of the organism to a particular environmental factor, such as SIgA.

Increased adherence of \textit{C. albicans} to buccal epithelial cells has benefits for both the host and the yeast. The host could potentially use the increase as a sequestering mechanism to restrain the yeast to epithelial cells, which are frequently sloughed. Therefore, the yeast is prevented from invading and causing infection. In the case of the yeast, adhering to epithelial cells has the obvious advantage of permitting colonization. However, adhering also prevents \textit{C. albicans} from being swallowed and destroyed in the digestive tract by the low pH. Interestingly, only two of the eight \textit{C. albicans} strains assayed displayed increased adherence. This could be attributed to the lack of a sufficient titer of \textit{C. albicans}-specific SIgA. Possibly, the titer of SIgA that was able to bind to \textit{C. albicans} was low enough to induce no change in adherence. To rule out this hypothesis, the experiments should be repeated using only \textit{Candida}-specific SIgA.

In summary, this research has fulfilled a well-defined, previously unanswered question, which was as follows: Can SIgA inhibit the frequency of germination in \textit{C. albicans}?

Additionally, the knowledge of adherence has been expanded. Adherence is highly dependent upon strain and increase in adherence is restricted to only a small portion of \textit{C. albicans} strains.
Applying *C. albicans*’ response to SIgA *in vivo* is more complex. SIgA is only a small part on the host response to virulence factors of *C. albicans*. Hosts sufficient in SIgA may still be susceptible to candidiasis. However, the susceptibility may be attributed to a deficiency in one of the other facets of the host’s complex immune response to microbial invasion.

Further research is needed in the areas of *C. albicans*’ germination and adherence. This study has answered basic questions concerning the impact of SIgA upon these virulence features. However, future studies are necessary to determine the mechanisms by which SIgA affects adherence and reduces germination. Knowing which epitope *Candida*-specific antibodies need to be directed against to decrease germination or affect adhesion is also needed to decipher a possible mode of action. Future experiments would focus on possible targets for *Candida*-specific antibodies that block the yeast-to-mycelium transition.

The results of this study have contributed to discerning the effects of one aspect of humoral immunity upon *C. albicans*. However, there is still a void in the current knowledge concerning SIgA’s impact on *C. albicans*’ germination and adherence. Possible future research might investigate the mechanism by which SIgA inhibits germination and affects adherence to epithelial cells. Strain variation was found to greatly impact response to SIgA and should be further investigated. The questions posed at the onset of this study have been answered, filling a portion of the large gap in current knowledge of humoral immunity and candidiasis.
REFERENCES


