Switch Phenotypes of *Candida albicans* Differ in the Oral Colonization of Immunosuppressed Balb/c Mice

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Purpose: Phenotypic switching is a reversible and heritable characteristic that provides *C. albicans* with the capacity to rapidly adapt to changes in its environment. The purpose of this study was to assess the ability of different switch phenotypes of a single strain of *Candida albicans* to colonize the oral mucosa of immunosuppressed Balb/c mice.

Material and Methods: Seventy two Balb/c mice were inoculated with one of four switch phenotypes of *Candida albicans*. During an experimental period of nine days, mice were swabbed daily for oral carriage and evaluated post mortem (day 9) for variability in phenotype and adhesion by SEM and TEM.

Results: Great variability was seen in the ability of switch phenotypes from a single strain of *Candida albicans* to colonize the oral mucosa of immunosuppressed mice. Very wrinkled and Ring had the highest levels of colonization followed by Smooth white and Heavily myceliated. TEM showed fimbrial attachment between yeast and epithelial cells and yeast themselves. SEM analysis showed differences in morphology of the switch phenotypes.

Conclusions: Some phenotypes of Candida albicans are better able to adhere to the oral mucosa of Balb/c mice. These differences may add to the ability of Candida albicans to survive under adverse conditions or evade the host immune response.

Key words: Candida, candidiasis, adhesion, switching

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INTRODUCTION

As the AIDS epidemic progresses into its third decade, the opportunistic infections caused by *Candida* species have continued to produce significant morbidity and mortality in this and other immunosuppressed populations. The most common forms of mucosal candidiasis are oropharyngeal and vaginal (Calderone, 2002) and studies have shown that 60-80% of HIV infected individuals will develop one or more candidal infections at some time during their illness (Hauman et al, 1993).

Phenotypic switching is a reversible and heritable characteristic that provides *C. albicans* with the capacity to rapidly adapt to changes in its environment (Kiraz et al, 2000; Odds, 1997; Odds and Merson-Davies, 1989). The observation of switching at sites of commensalism (Hellstein et al, 1993) and infection (Vargas et al, 2000a) and that switching can reversibly alter most virulence traits (Soll, 2002), including antifungal drug resistance (Kiraz et al, 2000; Vargas et al, 2000a), supports the contention that switching itself represents a putative virulence trait. Nevertheless, very limited *in vivo* experimental data exists that addresses differences in the ability of switch phenotypes to colonize host tissue.

The present study assesses the ability of different switch phenotypes of a single strain of *Candida albicans* to colonize the oral mucosa of immunosuppressed Balb/c mice. The experiments here support that switch phenotypes affect the initial stages of infection and that some phenotypes are more adept at colonization than others.

MATERIALS AND METHODS

Selection of Mice

Seventy-two 8 to 12 week old male Balb/c mice (Sprague-Dawley, Madison, WI) were used (4 groups of 16 mice each and one group of 8 mice (control)). The number of mice was chosen based on a previous power analysis. Approval for this project was obtained from the University of Iowa Animal Care Use Facility. All animals were housed in standard cages in groups of 4 and provided with food and water ad libitum.

C. albicans inoculum

Four switch phenotypes (Smooth White (SW), Very Wrinkled (VW), Ring and Heavily Myceliated (HM)) of a single strain (453) of *Candida albicans* originally isolated from an HIV-positive individual were used. Each phenotype was grown aerobically in liquid supplemental Lee's (Lee et al, 1975) medium to late log phase (2.5×10^7 cells/ml) at 25°C. Cells were washed twice in sterile 1× phosphate buffered saline (PBS) and resuspended in the same buffer at a cell density of 1×10^8 cells/ml. Switch phenotypes were verified by plating 10 μl of the suspension onto modified Lee's agar plates and incubating aerobically at 25°C for 7 days.

Immunosuppression of Mice and Candida Colonization

Before infection with *Candida* switch phenotypes, mice were immunosuppressed by pre-treating the animals 7 days with cyclophosphamide injected intraperitoneally (i.p.) at 150 mg/kg and 94 mg/kg cortisone on day 1 and 7.5 mg/kg cyclophosphamide and 94 mg/kg cortisone on days 2-7. After day 7, immunosuppression of mice was maintained with 7.5 mg/kg cyclophosphamide every other day and 94 mg/kg cortisone daily until the end of the experimental period of 18 days. The control group was divided in two which were not infected with *Candida* switch phenotypes. One-half (4 mice) were immunosuppressed as described and the other half (4 mice) received the same volume of saline injections. Blood samples were examined periodically to verify low white blood cell count associated with immunosuppression.

Oral Infection

Immunosuppressed mice were inoculated orally with 1×10^8 blastoconidia of one of the four phenotypes on days 8 and 9 using fibre-optic cotton tipped swab (Techspray Fiber Optic Swab, Techspray, Cedar Rapids, IA). The control mice were not inoculated with yeast. On day 10, infected mice were randomly divided into 2 groups of 8 each. One group received 30 mg/kg/day intraperitoneal injections of fluconazole (Pfizer, Limited, Sandwich, UK) once daily for 7 days starting 48 hours post-infection. The other group received the same volume of sterile saline solution. Infected mice were sampled for carriage of *C. albicans* phenotypes daily from day 10 until the termination of the study on day 18 using the fibre-optic cotton tipped swab. This was accomplished by inserting the swab into the mouth and firmly sampling the tongue and mucosal surfaces by rotating the swab for approximately five to ten seconds (Chakir et al, 1994; Samaranayake et al, 1998; Vargas et al, 2000b). On day 18, mice were sacrificed by cervical dislocation. For each switch phenotype group, the oral cavity of 1 mouse was scraped for wet mount cytology, 2 mice had the tongues removed for SEM and TEM and 3 mice were swabbed for carriage.

Assessment of Carriage

Beginning 24 hours post infection (day 10), samples were collected following methodology previously described (Vargas et al, 2000a; Vargas et al, 2004). Briefly, oral yeast samples were collected by passing a sterile cotton swab (Techspray Fiber Optic Swab, Techspray, Cedar Rapids, IA) several times across the oral mucosa. Immediately after sampling, each swab was placed in a sterile polypropylene test tube containing 200 μl PBS. Tubes were transported within two hours to the laboratory, where the suspensions were vigorously mixed for 30 seconds (using a vortexer) and 50 μl plated onto modified Lee's agar plates. Also, serial dilutions of the original suspension of 1/10 and 1/100 were plated. Plates were incubated aerobically at 25°C for 7 days; colonies on each plate were counted and scored for phenotype.

Microscopy

Excised tongues were sectioned longitudinally to obtain two halves with one half being prepared for scanning electron microscopy and the other half for transmission electron microscopy.

Light microscopy: Using a sterile tongue depressor, mucosal tissues were scraped and streaked on a glass slide for wet mount cytology. After fixation, the samples were stained with Periodic Acid Schiff reagent, dehydrated in graded alcohols, covered with ProPar Clearant, Permount mounting medium and a coverslip. Slides were viewed with an Olympus Provis AX70 light microscope (Olympus America, Inc., Melville, NY).

Scanning electron microscopy (SEM): Tongues were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer for two hours and post-fixed with 1% osmium

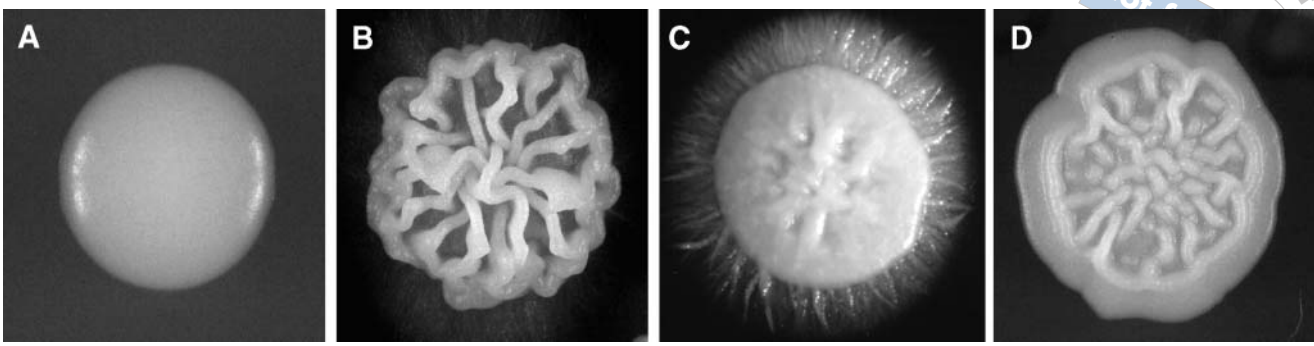

Fig. 1 Switch phenotypes isolated from a single strain of Candida albicans (453). (A) Smooth White (SW), (B) Very Wrinkled (VW), (C) Heavily Myceliated (HM) and (D) Ring.

tetroxide. Samples were dehydrated in graded acetone and hexamethyldisilizane (HMDS), sputter coated with palladium-gold and examined under a Hitachi S-4000 scanning electron microscope.

Transmission electron microscopy (TEM): Tongues were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for two hours. After post-fixation in 1% osmium tetroxide in buffer with 1.5% potassium ferrocyanide, samples were dehydrated in a graded series of acetone and embedded in Spurr's medium. Ultrathin sections were stained with uranyl acetate followed by lead citrate and observed under a Hitachi H-7000 transmission electron microscope.

Statistical Analyses

Data from the *in vivo* mouse study was analyzed with Repeated Measures (RM) ANOVA and Tukey post hoc tests.

RESULTS

Carriage of C. albicans Switch Phenotypes in Balb/c Mice

Immunosuppressed Balb/c mice were inoculated orally with one of the four phenotypes from strain 453 (SW, HM, VW or Ring) (Fig. 1) at a concentration of 1×10^8 over 2 days. Daily assessment of carriage rates for the four phenotypes in infected mice revealed differences when no fluconazole was present (Fig. 2A). On the first day of infection, a significant difference in inoculum retention is apparent. Both VW and Ring had the highest initial colonization ($p<0.05$) compared to SW and HM. Over the course of the nine day experiment, this initial difference in phenotype retention was maintained ($P<0.05$). Using RM ANOVA, we found that carriage rates for Ring and VW increased significantly throughout the 9 day period, whereas, SW maintained the original carriage and HM had a decrease in carriage by day 9 (Fig. 2A). Colonization by VW and Ring of immunosuppressed mice was significantly more than either SW or HM (Fig. 3).

To evaluate the effects of an antifungal agent on carriage rates of different switch phenotypes, mice received intraperitoneal injections of fluconazole at 30mg/kg/day beginning 48 hours after inoculation. A steady decline in carriage was seen for all four phenotypes (Fig. 2B) and by day five, carriage was virtually non-existent. Our previous studies have shown that the minimum inhibitory concentration of fluconazole for the four switch phenotypes of strain 453 range between 0.1 μg to 1 μg. Therefore, these results were not surprising.

Morphological Features of C. albicans Switch Phenotypes

The interactions of *C. albicans* strain 453 switch phenotypes with mouse epithelium were observed by light microscopy (Fig. 4), scanning electron microscopy (SEM) (Fig. 5) and transmission electron microscopy (TEM) (Fig. 6). Since we did not sacrifice any of the mice until the end of the experimental period, 10 post-infection samples were obtained.

After the mice representing all six groups (4 experimental and 2 control) were sacrificed, one from each group was randomly selected to have the mouth scraped for wet mount cytology (Fig. 4). Samples from the oral cavity were spread onto microscope slides and fixed with conventional PAS staining. Light microscopy at 40× magnification showed differences in adhesion type and density among the phenotypes. SW (Fig. 4A) cells could not be located in the wet mount samples. This may have been due to the decreasing carriage

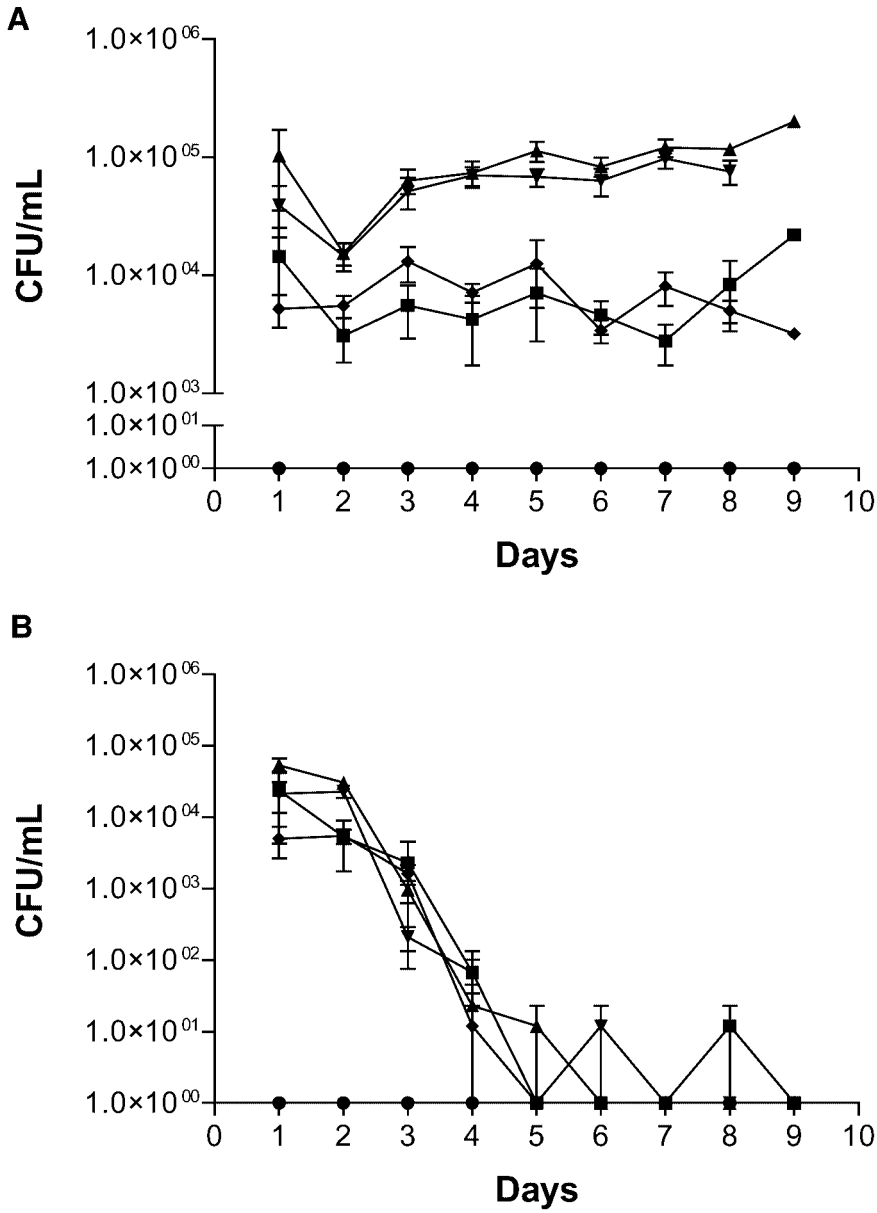

Fig. 2 (A) Carriage of Candida albicans switch phenotypes. RM ANOVA and ANOVA found significant differences in carriage among and between groups over time ($p<0.05$). (B) Retention of C. albicans switch phenotypes after fluconazole exposure. No differences were found in carriage rates over time for HM over the 9 day period when no fluconazole was present. After fluconazole administration, Ring and SW were carried through day 6 and 8 respectively with VW and HM being eliminated by day 4. • Control, ■ SW, ▲ VW, ◆ HM, ▼ Ring.

rates and the fact that our previous studies have shown SW to form very few hyphae. This may have affected the visibility/retention on the wet mount. VW showed predominantly hyphae and pseudohyphae (Fig. 4B). HM cells were made up mostly of long hyphae (Fig. 4C) and Ring had a combination of long and short hyphae (Fig. 4D).

Both uninfected and infected mouse tongues (from 2 mice in each group) were viewed under scanning electron microscopy (Figs. 5A-E). The control mouse tongue (Fig. 5A) showed uniform conically shaped filiform papilla and appeared normal. When mice were infected with the SW phenotype, SEM showed little to no adherent cells remaining on the surface (Fig. 5B). Although very few adherent cells were found, there was evidence of tissue sloughing in and around the papillae (Fig. 5B-arrows). Conversely, VW was observed in abundance, adhering to the interpapillary epithelium and projecting aerial hyphae (Fig. 5C-arrows). Here too, interpapillary tissue sloughing was apparent (Fig. 5C). Similarly, SEM examination of the HM phenotype shows an abundance of single cells interspersed with long hyphae and greatest adhesion in the interpapillary region (Fig. 5D). Figure 5E is representative of the Ring

phenotype. Although no yeast was visualized with SEM, much damage to red blood cell morphology as well as integrity of the epithelium was observed (Fig. 5E).

In order to assess the morphological characteristics of the interaction between the yeast cells and the epithelial cells, we prepared tongue samples for transmission electron microscopy (TEM). We found that the cell walls and cytoplasmic contents were similar for all phenotypes; therefore, the TEM sections have not been assessed by phenotype. In general, staining with osmium tetroxide showed the presence of a glycocalyx or fimbriae at the point of contact between yeast cells or between yeast and epithelial cells (Figs. 6A and 6B). In cases where the contact was between a yeast and epithelial cell, a deformation of the epithelial cell membrane could be seen (Fig. 6C). Once internalized, the yeast and/or hyphae lost the fimbrial layer (Fig. 6D).

DISCUSSION

Phenotypic switching has been implicated as a virulence factor for many years, yet very little *in vivo* data exists to confirm or contradict the occurrence and implications of switching in oral tissues. For this study, we used four switch phenotypes from a single strain of *C. albicans* that had originally been isolated from an HIV+ patient with the primary objective of evaluating "in vivo" differences in oral mucosal colonization among the switch phenotypes. By orally inoculating immunosuppressed Balb/c mice, we were able to follow colonization over a 9 day course of infection. Interestingly, we found significant differences not only among the phenotypes but within a phenotype over time. Both VW and Ring adhered very well to the oral mucosa. After an initial drop in colonization (day 2), both were back to their original inoculation concentration and surpassed this by day 9. On the other hand, HM colonization began declining by day 3. SW remained fairly constant after an initial drop in colonization until day 7. By day 8, it had reached its original inoculum concentration and by day 9 it had doubled. The differences seen in this study mimic those seen in another *Candida albicans* switching system, WO-1, that has been extensively studied and been shown to have differences in adhesion to buccal epithelial cells (Kennedy et al, 1988). More recently, Lan et al (2002) showed differential expression of the ALS and AAF1 adhesion related genes in WO-1. They found that under identical growth conditions, the white (W) phenotype displayed up-regulation of these genes when compared to the opaque (O) phenotype.

In order for an organism to cause disease, it must first adhere to the tissue. Our scanning electron microscopy studies revealed differences in morphology of the adherent phenotypes. Both VW and HM had numerous hyphae visible in the interpapillary regions of the filiform papilla. On the other hand, neither SW nor Ring could be readily discerned in the SEM sections. This could have been due to processing of these

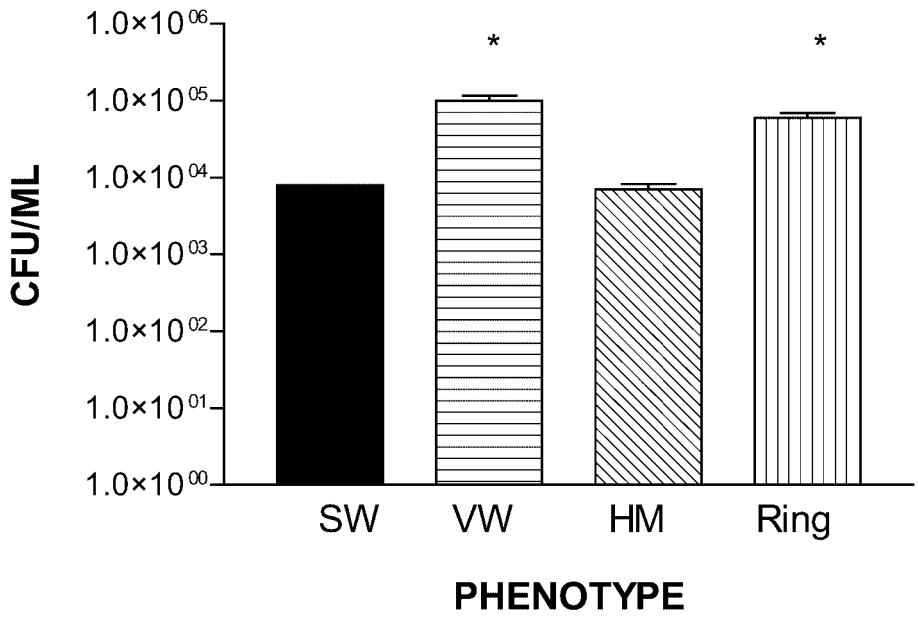

Fig. 3 Average carriage over the 9 day study period for SW, VW, HM and Ring. On average, carriage over the 9 day period was significantly different for VW and Ring (ANOVA, $p<0.05$).

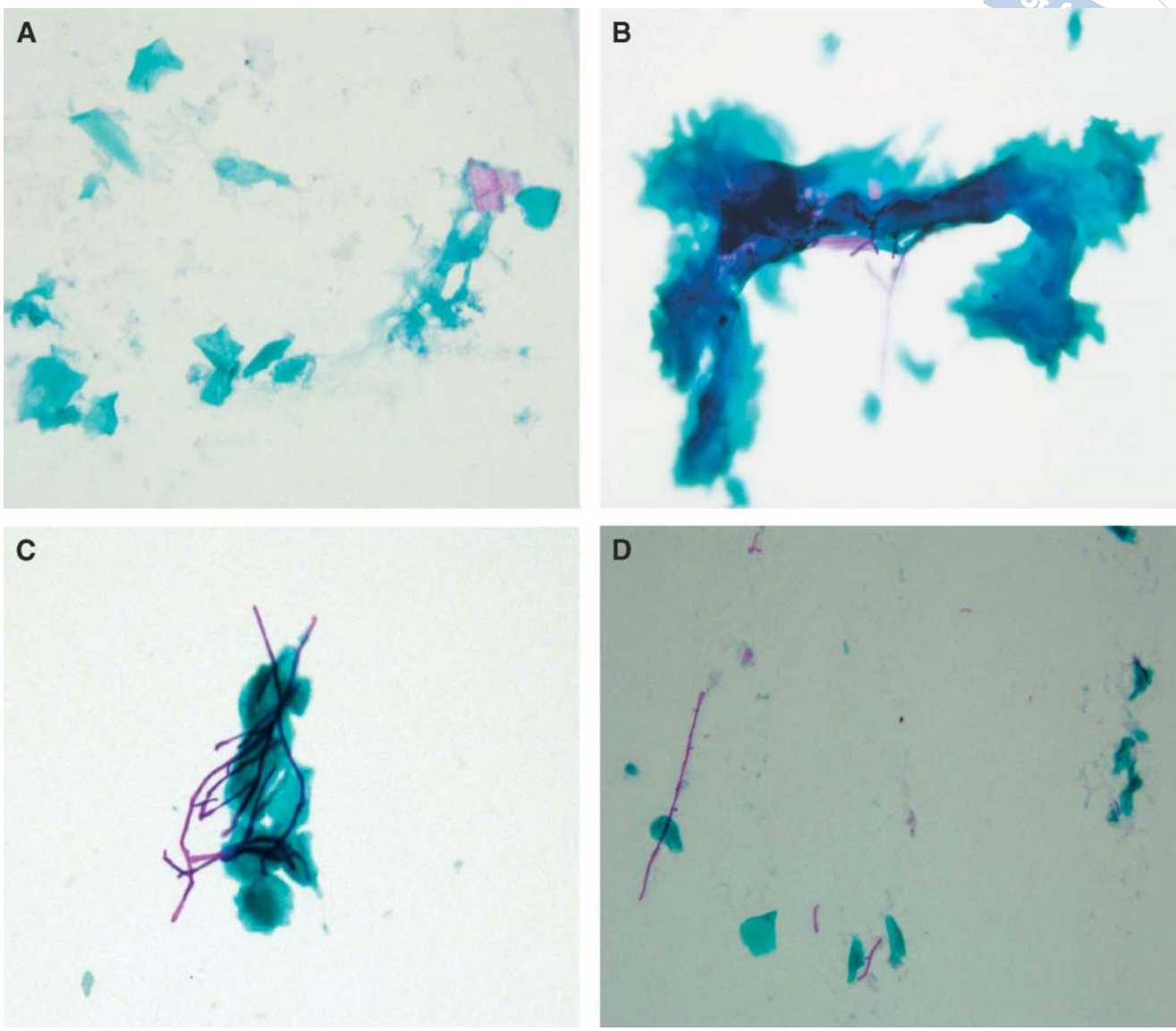

Fig. 4 Periodic acid Schiff (PAS) staining of C. albicans switch phenotypes on epithelial cells from infected Balb/C mice. (A) SW; no colonization was seen, (B) VW; note the large clumps of hyphae attached the epithelial cells, (C) HM; although colonization was less for this phenotype, when present, long hyphae were abundant and (D) Ring; cytological smear shows the presence of some hyphae, but more abundant were short hyphae and budding yeast.

phenotypes, as they have generally less hyphae than either HM or VW. Without the surface adhesiveness of the hyphae, these cells may have been lost during the fixation and dehydration methods. Nevertheless, tissue sloughing and cell damage can be seen in tongue sections colonized with SW and Ring. Supporting these observations, Villar et al (2004) used filamentation deficient mutants, and found that those cells that grew exclusively as blastospores or that formed pseudohyphae were much less able to adhere to epithelial cells than cells that formed true hyphae.

Moreover, these filamentation deficient mutants were also less capable of mounting a proinflammatory response from the host. Glycans on the cell surface of *Candida albicans* have been shown to play a major role in the adhesion of this organism to host cells. More specifically, mannan has been implicated as the active glycan (Masuoka, 2004). This can be seen as a "ruthenium-red stainable matrix surrounding the *Candida albicans* cell" (Masuoka, 2004). Barnes et al (1983) showed fibril connections between yeast cell surface and the endothelium of renal peritubular and glomeru-

lar capillaries. Similarly, our TEM sections showed *Candida* fimbriae adhering to fimbriae on the surface of epithelial cells and to fimbriae on other *Candida albicans* cells.

Evidence from this investigation and our previous studies on fluconazole uptake by switch phenotypes of strain 453 (Vargas et al, 2004) lend support to the role of phenotypic switching as an important virulence factor for *Candida albicans*. Moreover, the multifactorial nature of the disease process becomes even clearer. Since adhesion is the most constant aspect of the disease, elucidation of the host-organism interaction at this level may be an effective way of eliminating fungal disease.

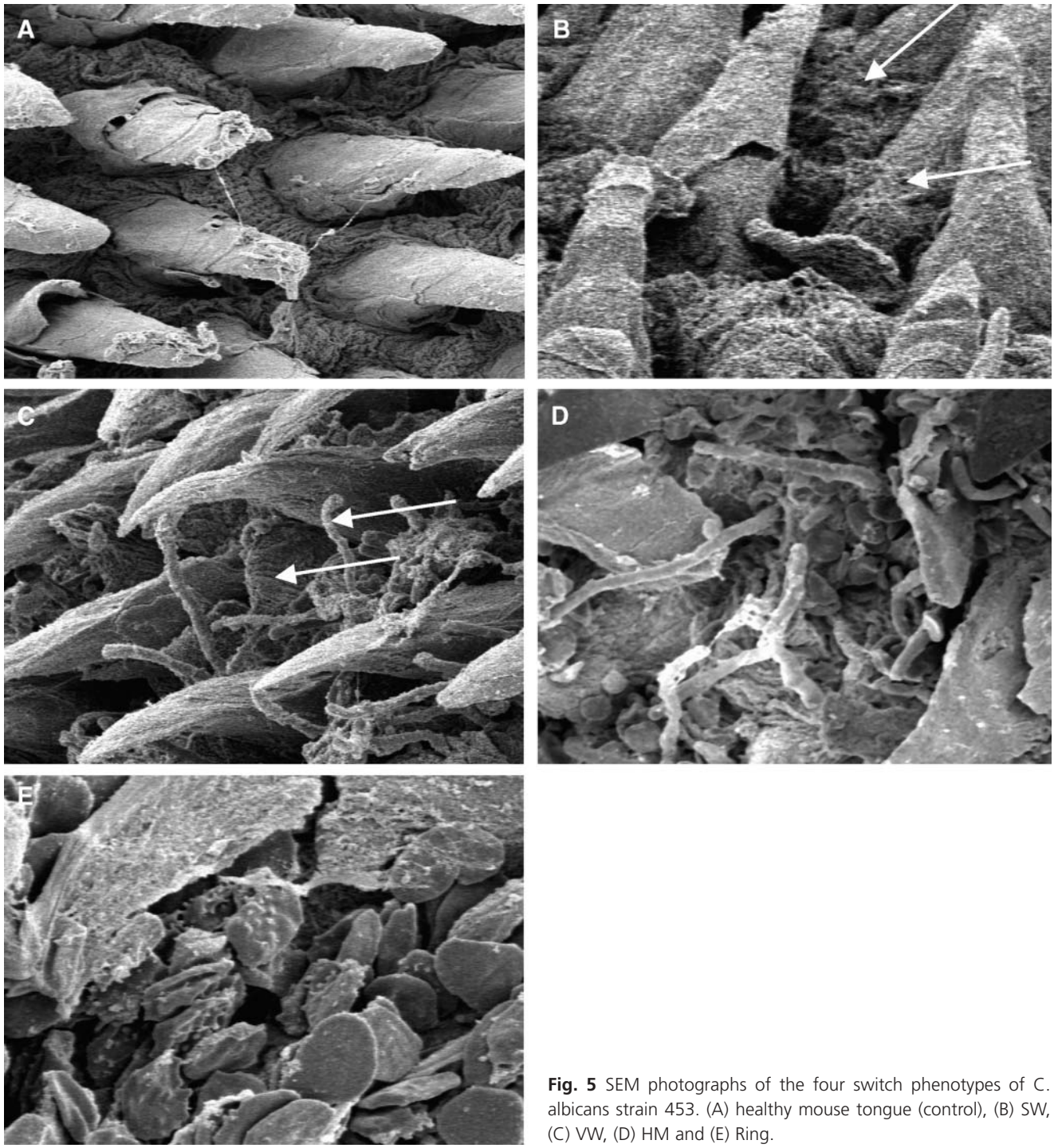

Fig. 5 SEM photographs of the four switch phenotypes of C. albicans strain 453. (A) healthy mouse tongue (control), (B) SW, (C) VW, (D) HM and (E) Ring.

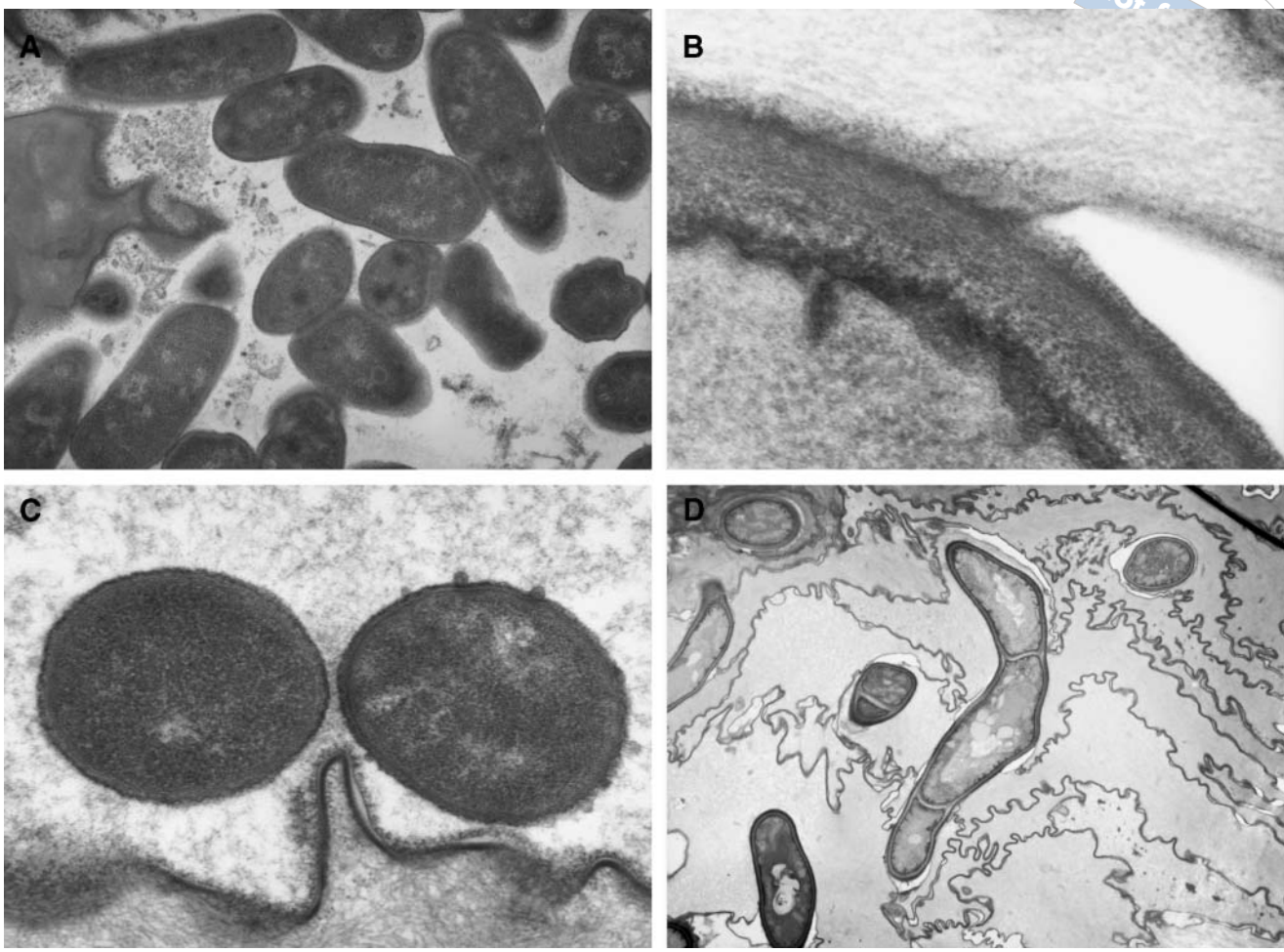

Fig. 6 TEM photographs of C. albicans adherence. (A) Multiple yeast cells adhering to each other. Note the fimbriae present at the point of contact between cells. (B) Closer magnification of the fimbriae present at the point of contact between yeast cells. (C) Yeast cells adherent to an epithelial cell. Note the deformation of the epithelial cell membrane that is in contact with the yeast fimbriae. (D) Internalized Candida cells. Note these cells are devoid of fimbriae once inside the epithelial cells.

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