Relationship Between Germination of *Candida albicans* and Increased Adherence to Human Buccal Epithelial Cells

LUCILLE H. KIMURA AND NANCY N. PEARSALL*

Department of Microbiology and Immunology, School of Medicine, University of Washington,
Seattle, Washington 98195

A strong correlation was shown between germination and increased adherence of *Candida albicans* to human buccal epithelial cells, indicating that germination or other changes in the fungi accompanying germination were responsible for enhanced adherence. Partial inhibition of germination by cysteine resulted in a comparably lower adherence. Preferential adherence of germinated fungi occurred in competition assays with nongerminated and germinated fungi. The enhanced adherence to human mucosal cells of germinated *C. albicans* could represent one mechanism contributing to the pathogenicity of the organism.

The adherence of bacteria to epithelial cells is an important initial step in the colonization of mucous membranes (7). This has been shown for a variety of ecological systems, including streptococci in the oral cavity (8), *Vibrio cholerae* in the intestinal tract (6), toxin-producing *Escherichia coli* in the intestinal tract (11), *Neisseria gonorrhoeae* in the genital tract (16), and many others. We showed that the opportunistic fungus, *Candida albicans*, that colonizes mucous membranes also adheres to epithelial cells from the human oral mucosa (13).

Liljemark and Gibbons (15) observed the attachment of *C. albicans* to oral cells from rodents and showed that adherence was greater with epithelial cells from germ-free animals than with cells from conventional animals, suggesting that indigenous bacterial flora may interfere with fungal attachment and colonization.

Our studies (13) on the attachment of *C. albicans* to human buccal epithelial cells showed that adherence occurred in the presence of saliva or in phosphate-buffered saline (PBS) and was greater at 37°C than at 25°C. Enhanced adherence occurred under various conditions that favored germination of the yeasts, such as incubation at 37°C in saliva or in tissue culture medium 199 (TC 199). After the fungi had germinated, they could be killed and would still manifest enhanced adherence. It was postulated that this enhanced adherence resulted from changes in the fungi that occur during germination.

The experiments reported here were designed to further test the hypothesis that germination of the fungi leads to enhanced adherence of *C. albicans* to human buccal epithelial cells.

MATERIALS AND METHODS

Adherence media. Adherence tests were carried

out in 0.01 M PBS (isotonic buffer containing sodium phosphate and sodium chloride at pH 7.0) or in TC 199 (GIBCO Laboratories, Santa Clara, Calif.). The TC 199 was adjusted to pH 7.0 with sodium bicarbonate and did not contain serum or antibiotics.

Epithelial cells. Buccal epithelial cells were collected from healthy human subjects by gently rubbing the inside of the cheeks with sterile swabs which were then agitated in 12 ml of PBS. Epithelial cells were washed twice in PBS to remove unattached microorganisms and were resuspended in PBS or TC 199 at 2×10^5 cells per ml. Epithelial cells from several donors were pooled for each adherence test. Washed epithelial cells usually had no attached fungi before the adherence test.

Yeast cell suspensions. Recent isolates of *C. albicans* from the oral cavity of a healthy human subject were identified by standard techniques (20) and maintained on Sabouraud glucose agar stored at 4°C. The strain designated N-1-5 was used for most of these studies. For the adherence tests, yeasts were grown for 18 to 24 h at 37°C on Sabouraud glucose agar, transferred to Sabouraud glucose broth, and incubated at 37°C for 18 to 24 h. The fungi, which were virtually all in the budding yeast phase, were washed twice in PBS and suspended in the appropriate test medium at 10^8 cells per ml. Procedures for inducing germination in some experiments are described in the text.

Adherence assay. The adherence assay described by Kimura and Pearsall (13) was used. Briefly, 0.2 ml of epithelial cells and 0.2 ml of fungi were mixed in tubes and incubated on a rocker at 37°C for 1 to 3 h. Control tubes contained epithelial cells and PBS or another diluent. After incubation, the epithelial cells were collected on 12- μ m-pore size polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.) and washed with 70 ml of PBS to remove unattached fungi. The washed epithelial cells on the filters were air dried, fixed with absolute methanol, and stained with Gram crystal violet. At least 100 epithelial cells on the filters were counted, and the numbers of fungi adhering per 100 epithelial cells were determined. All filters were coded and read "blind." The presence of germ tubes was assessed visually.

RESULTS

Comparison of direct counts of germinated and nongerminated *C. albicans* attached to epithelial cells in a mixture or free in suspensions without epithelial cells. Direct counts of the numbers of germinated and nongerminated forms of *C. albicans* in suspensions either with or without epithelial cells were compared. The results (Table 1) showed that the percentage of germinated forms attached to epithelial cells exceeded that of germinated forms in similar suspensions without epithelial cells. For example, at a fungal cell concentration of 5×10^7 /ml, at 1 h 19% of the attached fungi were germinated, compared with 1% of germinated fungi in suspensions without epithelial cells. At 2 h, 58% of adherent fungi were germinated, compared with only 20% of those in suspensions without epithelial cells. These results indicate that germinated *C. albicans* attach selectively and to a greater extent than do nongerminated yeasts.

Effects of inhibiting germination on adherence. Our previous results indicated that inhibiting germination in TC 199 by incubating the fungi at 25°C resulted in a significantly lower adherence than at 37°C (13). These findings could have resulted from temperature effects other than from the inhibition of germination; therefore, another approach was used to inhibit germination. Dabrowa et al. (1) reported that 0.01 M cysteine inhibits the germination of *C. albicans*; therefore, adherence assays were carried out in TC 199 containing 0.01 M cysteine. Figure 1 shows the results of an experiment in which fungi were incubated with epithelial cells at a ratio of 500:1. Germination and adherence were slightly inhibited at 2 h in medium contain-

FIG. 1. Adherence of viable *C. albicans* to buccal epithelial cells in TC 199 in the presence and absence of 0.01 M cysteine.

ing 0.01 M cysteine in 37°C, and both were profoundly inhibited in TC 199 at 25°C. The adherence in this experiment was expressed as the percentage of epithelial cells having more than 10 *C. albicans* attached because the numbers of attached fungi on many epithelial cells were too high to permit accurate counting. Another experiment was done with 0.05 M cysteine to achieve a greater inhibition of germination and a lower ratio of fungi to epithelial cells (100:1) to facilitate counting; the results are presented in Table 2. In the presence of 0.05 M cysteine, both germination and adherence were markedly lower by 150 min than in medium without cysteine. Germination was 29% in cysteine compared with 58% in medium without cysteine, and adherence was significantly lower in cysteine ($P < 0.05$). Thus, experiments using cysteine to inhibit germination showed a positive correlation between the extent of germination and the degree of adherence.

Competition assays with germinating and nongerminating fungi. Germinating fungi were prepared by incubating 10^6 *C. albicans* yeast cells per ml in TC 199 at 37°C for 80 min. At this time $79 \pm 2\%$ of the fungi had short germ tubes. Nongerminating fungi were obtained by incubating 10^6 *C. albicans* yeast cells per ml for 80 min either in TC 199 at 25°C or in Hanks balanced salt solution (pH 7.0) at 37°C. All preparations were rapidly cooled in an ice bath after incubation, (to prevent the formation of long germ tubes which would interfere with

TABLE 1. Adherence of nongerminated and germinated forms of *C. albicans* to buccal epithelial cells in TC 199

Fungi per ml	Time (min)	Attached fungi per 100 epithelial cells $\pm$ SD ^a (% of total attached fungi)		% Germinated fungi in suspension ^b
		Nongerminated	Germinated	
5×10^7	0	13 $\pm$ 4 (100)	0 (0)	0
	60	328 $\pm$ 115 (81)	75 $\pm$ 38 (19)	1 $\pm$ 1
	120	440 $\pm$ 123 (42)	605 $\pm$ 169 (58)	20 $\pm$ 6
10^7	0	6 $\pm$ 3 (100)	0 (0)	0
	30	59 $\pm$ 27 (100)	0 (0)	0
	60	230 $\pm$ 88 (90)	26 $\pm$ 8 (10)	4 $\pm$ 2
	90	261 $\pm$ 41 (54)	219 $\pm$ 31 (46)	22 $\pm$ 4
	120	154 $\pm$ 50 (31)	340 $\pm$ 49 (69)	58 $\pm$ 4
	150	131 $\pm$ 42 (13)	848 $\pm$ 150 (87)	80 $\pm$ 4

^a Means of triplicate determinations $\pm$ standard deviation (SD).

^b Fungi were incubated in TC 199 in the absence of epithelial cells.

the assay), washed twice in cold PBS, and adjusted to 2×10^7 cells per ml. Suspensions containing 0, 20, 40, 60, and 80% germinating fungi were prepared in PBS by mixing germinated with nongerminated cells. These suspensions were then added to buccal epithelial cells at a ratio of 100 fungi to 1 epithelial cell, and the mixtures were incubated at 25°C for 30 min.

Figure 2 shows that increases in total numbers of attached fungi paralleled increased proportions of germinated fungi and were not related to the proportions of nongerminated fungi present. In experiment 1, the nongerminated fungi

TABLE 2. Adherence of *C. albicans* to buccal epithelial cells in TC 199 with cysteine at 37°C

Cysteine	Attached fungi per 100 epithelial cells $\pm$ SD ^a (% of fungi with germ tubes) at:		
	30 min	90 min	150 min
0	79 $\pm$ 15 (0)	381 $\pm$ 137 (28)	760 $\pm$ 151 (58)
0.01 M	116 $\pm$ 64 (0)	435 $\pm$ 41 (15)	684 $\pm$ 62 (75)
0.05 M	65 $\pm$ 16 (0)	367 $\pm$ 172 (4)	411 $\pm$ 79 ^b (29)

^a Means of triplicate determinations $\pm$ standard deviation (SD).

^b At 150 min, adherence with 0.05 M cysteine was significantly lower than adherence without cysteine ($P < 0.05$) and with 0.01 M cysteine ($P < 0.02$).

FIG. 2. Competition adherence assay with germinated and nongerminated *C. albicans*. In experiment 1 nongerminated fungi were treated with TC 199 at 25°C. In experiment 2 nongerminated fungi were treated with Hanks balanced salt solution at 37°C. In both experiments, germinated fungi were treated with TC 199 at 37°C.

were prepared in TC 199 at 25°C, and in experiment 2, the nongerminated fungi were prepared in Hanks balanced salt solution at 37°C. The results of both experiments showed that the numbers of attached germinated forms increased directly and dramatically with the numbers of germinated forms, rising from about 450 at 20% to about 1,200 at 80% of germinated fungi. Although there was a slight increase in numbers of attached nongerminated fungi, the difference was small, increasing from about 125 to about 250 as the percentage of nongerminated forms increased from 20 to 100%. The conclusion is that selective attachment of germinated fungi occurred.

Adherence of nongerminating *C. albicans*. When *C. albicans* was incubated at 10^6 to 10^7 cells per ml in TC 199 at 37°C, usually about 3 to 5% of the cells did not germinate, even after long periods of incubation. These nongerminating cells could be isolated from germinating fungi by the following method. TC 199 was inoculated with the stock strain of *C. albicans* and incubated overnight at 37°C. The culture, which contained primarily mycelial-phase cells and a few nongerminated fungi, was filtered over 12- μ m-pore size polycarbonate filters. Over 99% of the cells in the filtrate were nongerminated fungi. These cells were harvested by centrifugation and transferred to TC 199 for overnight incubation at 37°C and were then filtered again to remove the few mycelial elements that had formed. When these nongerminated cells were grown in Sabouraud glucose broth for a few days and then placed in TC 199, many of them germinated, suggesting that the numbers of fungi capable of germinating in TC 199 had quickly increased in Sabouraud glucose broth. When the nongerminated cells were grown in TC 199 instead of in Sabouraud glucose broth, approximately 99% of these cells remained in the yeast form, whereas the few cells that germinated formed long mycelial elements.

For testing the adherence of the nongerminating fungi, the cells were incubated overnight in TC 199 at 37°C, filtered to remove the few filaments that formed, washed, and suspended in PBS. An abundantly germinating fungal preparation was obtained from the same original stock of *C. albicans* by growing the fungi in Sabouraud glucose broth overnight, washing the cells in PBS, and incubating them at 10^7 cells per ml in TC 199 at 37°C. Incubation was terminated after 40 min when a majority (72%) of the fungi had germinated and had germ tubes that were shorter than approximately 10 μ m, a size which still enabled the fungi to pass through the filter during the washing step of the adherence assay. The fungi were washed and sus-

pended in cold PBS. Fungal cell mixtures were prepared containing the following percentage ratios of abundantly germinating to nongerminating fungi: 100:0, 75:25, 50:50, 25:75, and 0:100. These mixtures were added to buccal epithelial cells in PBS at a ratio of 100 fungi to 1 epithelial cell and incubated for 30 min at 25°C.

The results indicated that total counts of adherent fungi increased directly with an increase in the percentage of abundantly germinating fungi (Fig. 3). In addition, this adherence closely followed the adherence of germinated forms. The adherence of nongerminated forms fluctuated slightly with the changing percentages and did not show a consistent trend. This fluctuation and the slight increase in adherent nongerminated fungi observed when only the abundantly germinating preparation was tested probably resulted from the nongerminating cells in this preparation (approximately 28%). The results of this experiment again demonstrated a correlation between the extent of germination and the degree of adherence.

DISCUSSION

The results presented here show a strong correlation between germination and increased adherence of *C. albicans* to buccal epithelial cells, indicating that germination or other changes in the fungi accompanying germination were responsible for enhanced adherence. Incubation of viable *C. albicans* in TC 199 at 37°C, a condition that permitted germination, led to increased adherence. Partial inhibition of germination by

cysteine resulted in a comparably lower adherence. Preferential adherence of germinated fungi occurred in competition assays with nongerminated and germinated fungi. In all of these experiments, both the germ tubes and adjoining yeast cell walls could serve as attachment sites. Further studies by electron microscopy indicate that germ tubes attached more frequently than did other portions of the fungi (L. H. Kimura, J. C. Lata, and N. N. Pearsall, manuscript in preparation).

Other evidence of the greater adherence of germinated rather than nongerminated fungi comes from studies with human neutrophils by Diamond and Krzesicki (2) and Diamond et al. (3). They reported that germinated *C. albicans* attached to neutrophils in Hanks balanced salt solution in the absence of serum; contact between nongerminated yeast forms and neutrophils was low, even when the numbers of yeast cells were increased to account for the difference in fungal surface area. Hyphal forms of *Aspergillus fumigatus* and *Rhizopus oryzae* also attached to neutrophils, whereas encapsulated yeast forms of *Cryptococcus neoformans* did not attach to neutrophils in the absence of serum. It was suggested that the capacity of fungi to interact with leukocytes in the absence of serum reflected a general property of large forms of fungi (3).

Preliminary studies with other *Candida* species also showed enhanced adherence of fungi that had germinated in TC 199 (T. Mueller and N. N. Pearsall, unpublished data). In these studies, *C. albicans*, *Candida tropicalis*, and *Candida stellatoidea* germinated in TC 199 and adhered to a significantly greater extent to buccal epithelial cells in TC 199 than did nongerminating fungi, including *Candida guilliermondii* and *Saccharomyces cerevisiae*.

The greater adherence of germinated forms of *Candida* to epithelial cells may explain why some species colonize mucosal areas to a greater extent than other species. Young et al. (25) found that *C. albicans* accounted for 93.8% of the yeasts isolated from normal mouths, followed by *C. tropicalis* (2.1%) and *C. stellatoidea* (1.4%); other species of *Candida* were isolated much less frequently. *C. albicans*, *C. tropicalis*, and *C. stellatoidea* germinated in serum or TC 199; in other media, such as peptone, only *C. albicans* formed germ tubes (12). It would be of interest to compare the relative adherence of *Candida* species to determine whether germinating species adhere more readily than nongerminating species under conditions comparable to those in which colonization occurs.

The controversy over the relative virulence of yeast and mycelial forms of *C. albicans* has not

FIG. 3. Competition adherence assay with abundantly germinating and poorly germinating *C. albicans*.

been settled. Evidence has been presented suggesting that germination and filament formation are not necessary for pathogenesis (4, 5, 17, 21, 23), and other evidence supports the concept that the mycelial form is more virulent than the yeast form (9, 10, 19, 24). Certainly, mycelial forms are almost always found in lesions of candidiasis (9, 14, 18, 24) and are commonly observed in the oral cavity of persons with oral candidiasis (22). Our finding that germinated forms exhibit enhanced adherence to human mucosal cells could represent one mechanism contributing to the pathogenicity of the organism.

ACKNOWLEDGMENTS

This work was supported by the Center for Research in Oral Biology, Public Health Service grant DE-02600 from the National Institute of Dental Research.

We thank Paula R. Sundstrom for excellent technical assistance and for stimulating suggestions.

LITERATURE CITED

1. Dabrowa, N., S. S. S. Taxer, and D. H. Howard. 1976. Germination of *Candida albicans* induced by proline. *Infect. Immun.* 13:830-835.
2. Diamond, R. D., and R. Krzesicki. 1978. Mechanisms of attachment of neutrophils to *Candida albicans* pseudohyphae in the absence of serum, and of subsequent damage to pseudohyphae by microbicidal processes of neutrophils in vitro. *J. Clin. Invest.* 61:360-369.
3. Diamond, R. D., R. Krzesicki, and W. Jao. 1978. Damage to pseudohyphal forms of *Candida albicans* by neutrophils in the absence of serum in vitro. *J. Clin. Invest.* 61:349-359.
4. Eisman, P. C., S. G. Geftic, and R. L. Mayer. 1953. Virulence in mice of colonial variants of *Candida albicans*. *Proc. Soc. Exp. Biol. Med.* 82:263-264.
5. Evans, Z. A., and D. N. Mardon. 1977. Organ localization in mice challenged with a typical *Candida albicans* strain and a pseudohyphal variant. *Proc. Soc. Exp. Biol. Med.* 155:234-238.
6. Freter, R., and G. W. Jones. 1976. Adhesive properties of *Vibrio cholerae*: nature of the interaction with intact mucosal surfaces. *Infect. Immun.* 14:246-256.
7. Gibbons, R. J. 1977. Adherence of bacteria to host tissue, p. 395-406. *In* D. Schlessinger (ed.), *Microbiology—1977*. American Society for Microbiology, Washington D.C.
8. Gibbons, R. J., and J. van Houte. 1971. Selective bacterial adherence to oral epithelial surfaces and its role as an ecological determinant. *Infect. Immun.* 3:567-573.
9. Hill, D. W., and L. P. Gebhardt. 1956. Morphological transformation of *Candida albicans* in tissues of mice. *Proc. Soc. Exp. Biol. Med.* 92:640-644.
10. Iannini, P. B., G. D. Arai, and F. M. LaForce. 1977. Vascular clearance of blastospore and pseudomycelial phase *Candida albicans*. *Sabouraudia* 15:201-205.
11. Jones, G. W., and J. M. Rutter. 1972. Role of the K88 antigen in the pathogenesis of neonatal diarrhea caused by *Escherichia coli* in piglets. *Infect. Immun.* 6:918-927.
12. Joshi, K. R., J. B. Gavin, and D. A. Bremmer. 1973. The formation of germ tubes by *Candida albicans* in various peptone media. *Sabouraudia* 11:259-262.
13. Kimura, L. H., and N. N. Pearsall. 1978. Adherence of *Candida albicans* to human buccal epithelial cells. *Infect. Immun.* 21:64-68.
14. Lehner, T. 1966. Classification and clinico-pathologic features of *Candida* infections in the mouth, p. 119-137. *In* H. I. Winner and R. Hurley (ed.), *Symposium on Candida infections*. Churchill-Livingston, Edinburgh.
15. Liljemark, W. F., and R. J. Gibbons. 1973. Suppression of *Candida albicans* by human oral streptococci in gnotobiotic mice. *Infect. Immun.* 8:846-849.
16. Mårdh, P.-A., and L. Weström. 1976. Adherence of bacteria to vaginal epithelial cells. *Infect. Immun.* 13:661-666.
17. Mardon, D. N., J. L. Gunn, and E. Robinette. 1975. Variation in the lethal response in mice to yeast-like and pseudohyphal forms of *Candida albicans*. *Can. J. Microbiol.* 21:1681-1687.
18. Pearsall, N. N., and D. Lagunoff. 1974. Immunological responses to *Candida albicans*. I. Mouse-thigh lesion as a model for experimental candidiasis. *Infect. Immun.* 9:999-1002.
19. Russell, C., and J. H. Jones. 1973. The effects of oral inoculation of the yeast and mycelial phases of *Candida albicans* in rats fed on normal and carbohydrate rich diets. *Arch. Oral Biol.* 18:409-412.
20. Silva-Hutner, M., and B. H. Cooper. 1974. Medically important yeasts, p. 491-507. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), *Manual of clinical microbiology*, 2nd ed. American Society for Microbiology, Washington, D.C.
21. Simonetti, N., and V. Strippoli. 1973. Pathogenicity of the Y form as compared to M form in experimentally induced *Candida albicans* infections. *Mycopathol. Mycol. Appl.* 51:19-28.
22. Walker, D. M. 1975. Candidal infection of the oral mucosa, p. 467-505. *In* A. E. Dolby (ed.), *Oral mucosa in health and disease*. Blackwell Scientific Publications, Oxford.
23. Winsten, S., and T. J. Murray. 1956. Virulence enhancement of a filamentous strain of *Candida albicans* after growth on media containing cysteine. *J. Bacteriol.* 71:738.
24. Young, G. 1958. The process of invasion and the persistence of *Candida albicans* injected intraperitoneally into mice. *J. Infect. Dis.* 102:114-120.
25. Young, G., H. G. Resca, and M. T. Sullivan. 1951. The yeasts of the normal mouth and their relation to salivary acidity. *J. Dent. Res.* 30:426-430.