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NOTES

Variability in Expression of Cell Surface Antigens of *Candida albicans* during Morphogenesis

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The location and expression of two different cell surface antigens on germinating and nongerminating *Candida albicans* cells was examined by using transmission electron microscopy after labeling with monoclonal antibodies (H9 or C6) and immunocolloidal gold. Immunodeterminant expression of the two carbohydrate antigens was followed from early germination events through 20 h of development. The determinant detected by H9 antibody, which was initially lost from the mother cell surface and preferentially expressed only on hyphae during the first 4 h of germination, reappeared on the mother cell by 20 h, whereas the antigen detected by antibody C6 was continually expressed on mother cells and germ tubes throughout germination.

Candida albicans is a dimorphic fungus and an interesting organism because of the enigmas which surround both its human pathogenic and morphogenetic mechanisms (8, 17). To address these questions requires characterization of the antigenic mosaic surface of the fungus. Yeast cell walls are chiefly composed of glucan, mannan, protein, and small amounts of lipids (1, 11). The chemical and physical analyses of yeast and hyphal cell walls have demonstrated only quantitative but not qualitative differences (3, 6, 7, 11). Cell surface glycoproteins have been implicated in the adherence of yeast cells to mammalian host cells (12, 13, 22, 26). Although surface antigens make initial contact with host defense mechanisms, these antigens are not equally expressed among *Candida* strains (9, 15, 24, 27) and vary during growth of yeast-phase cells in vitro (4, 5) and in vivo (19). This dynamic expression of antigens and the lack of definition of cell surface composition during growth may explain the controversy regarding the role of antibody in disease (2, 16, 18) and other vague understandings of host-parasite relationships in candidiasis. We (4; D. L. Brawner and J. E. Cutler, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, F68, p. 375) and others (20, 25, 26) have demonstrated that *C. albicans* cells express antigens dynamically as a function of their metabolic or morphological states. Two specific yeast-phase determinants have been followed during growth of *C. albicans* and other yeasts (4, 5), but studies of antigenic expression in hyphal forms have been largely confined to the use of polyclonal antisera specific for cytoplasmic antigens (14, 23). The few comparative studies of surface antigens found in hyphal forms have been done with absorbed polyclonal antisera (23, 25).

Previous studies from our laboratory have shown that antigen expression in yeast-phase cells varies with growth and environmental conditions (4; Brawner and Cutler, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, F68, p. 375). We now present evidence that *C. albicans* also expresses surface antigens dynamically during hyphal development. We show that at least one antigen is invariantly expressed on both mother cells and germ tubes whereas another is found intermittently on mother cells or germ tubes during growth.

C. albicans 105 was used throughout the study and was chosen for its germinative ability. Germ tube tests were consistently positive (92 to 98% by 3.5 h) in either human serum or a defined germination medium, GM-2 (10). For immunoelectron microscopy (IEM) studies the organisms were grown to stationary-phase yeast forms in 2% glucose (J. T. Baker Chemical Co., Phillipsburg, N.J.)–0.3% yeast extract (BBL Microbiology Systems, Cockeysville, Md.)–1.0% peptone broth (Difco Laboratories, Detroit, Mich.) (GYEPB) before germ tube induction in GM-2 (see below). The hybridoma techniques have been previously described (4), and monoclonal antibodies H9 and C6, both immunoglobulin M (IgM) agglutinins, have been characterized (4, 5). The antigens which react specifically with monoclonal antibodies C6 and H9 (AgC6 and AgH9, respectively) have been characterized previously (4, 5).

Normal mouse ascitic fluid, induced by the injection of NS-1 myeloma cells into Pristane-treated mice, was diluted 1:4 in 0.01 M phosphate-buffered saline (PBS) and absorbed three times with both 10^{10} GYEPB-grown and 10^{10} GM-2-grown *C. albicans* 105 cells at 4°C (2 h per absorption). The 1:4-diluted, absorbed ascitic fluid was subsequently diluted 2.5 times in 0.15 N saline to a final working dilution of 1:10. Cells were prepared for IEM as described below, and the results using absorbed, diluted ascitic fluid were compared with those obtained using unabsorbed fluid.

Germinating cells of *C. albicans* nonspecifically bound colloidal gold-labeled antibody and autoagglutinated despite adsorption of the cells with 20% fetal bovine serum (Hyclone Laboratories, Logan, Utah) or 20% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). Nonspecific binding was eliminated beyond background levels, however, by pretreating cells with trypsin. One milliliter of trypsin solution (0.05% trypsin [Sigma] in Earle balanced salt solution, pH 7.6) was added to washed cell pellets (10^8 cells) of germinating and nongerminating cells which had been harvested after 0.5, 1, 2, 4, and 24 h of growth in GM-2. Control cells were exposed to Earle balanced salt solution without

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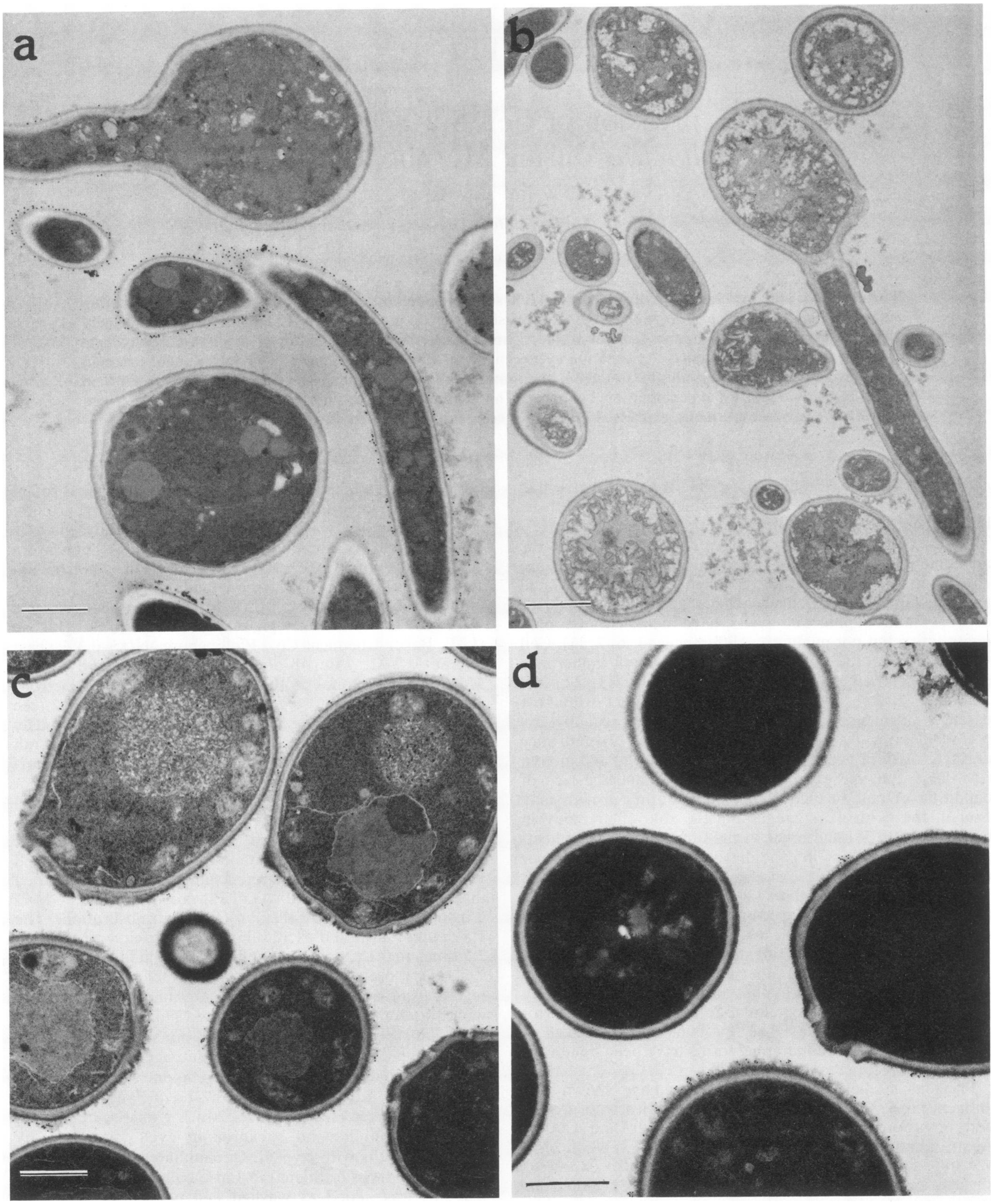

FIG. 1. Effects of trypsin treatment on specific and nonspecific binding of colloidal gold-conjugated antibody. Shown are (a) nontrypsinized germinating cells at 2.5 h of germination, (b) germ tubes from cells pretreated with trypsin before reactions with normal ascitic fluid and colloidal gold-conjugated antibody, and yeast-phase cells grown for 2.5 h which were either trypsin treated (c) or untreated (d). Bars, 1 μm.

TABLE 1. Detection of surface antigen of *C. albicans* by indirect IEM

Time (h)	Cell	Response of germinating cells (% responders)[a]		Response of nongerminating cells[b] (% responders)	
		Antibody H9	Antibody C6	Antibody H9	Antibody C6
0.25		2+ (75)	2+ (99)	2 to 3+ (75)	3 to 4+ (25)[c]
0.50		2+ (90)	2 to 3+ (100)	ND[d]	ND
1.0	Mother cell	− (100)	2 to 3+ (100)	3 to 4+ (75)	1 to 2+ (100)
	Germ tube	2+[e] (100)	1 to 2+[a] (100)		
2.0	Mother cell	− (100)	3+ (100)	2 to 3+ (95)	1 to 2 (100)
	Germ tube	2+ (100)	1 to 2+ (100)		
4.0	Mother cell	− (99)	3+ (100)	2 to 4+ (75)	1 to 3+ (100)
	Germ tube	1 to 2+ (100)	1 to 2+ (100)		
20	Mother cell	4+ (100)	ND	ND	ND
	Germ tube	2+ (100)			

[a] IEM evaluation: 1+, rare (<10 particles of gold bound per cell); 2+, scanty (10 to 20 particles of gold bound per cell); 3+, moderate (20 to 100 particles gold bound per cell); 4+, heavy (>100 particles of gold bound per cell); −, usually 0 gold particles per cell, rarely up to three. Numbers within parentheses indicate the percentage of cells displaying the indicated label density.

[b] Cells treated with morphogenic autoregulatory substance or a high yeast cell concentration to suppress germination.

[c] Remaining 75% were − or 1+.

[d] ND, Not done.

[e] Most heavily labeled at apices.

trypsin. The enzyme-cell mixtures were incubated at 37°C for 30 min with frequent mild agitation. After 0.5 h, 1.0 ml of ice-cold RPMI 1640 tissue culture medium (GIBCO Laboratories, Grand Island, N.Y.) containing 12% fetal bovine serum was added; the cells were immediately pelleted, washed twice in cold 0.01 M PBS, and then treated as described below for immunoelectron microscopy. Trypsinization of germinating cells eliminated autoagglutination after 1, 2, and 4 h of germination and reduced the degree of autoagglutination after 20 h of germination. Germinating cells which had consistently bound gold label nonspecifically without trypsin treatment (Fig. 1a) bound very little, if any, gold nonspecifically after trypsin treatment (Fig. 1b).

Nongerminating cells did not autoagglutinate or nonspecifically bind gold-labeled antibody. However, to establish that trypsin treatment did not alter the ability of antibody to bind AgH9 and AgC6, trypsin-treated (as above) nongerminating cells were reacted with monoclonal antibody and conjugated secondary antibody as described below, and the amount of gold conjugate bound was compared with that in nontrypsinized control cells. The binding patterns of the specific monoclonal antibodies were identical on trypsinized and nontrypsinized yeast cells; both cell populations when reacted with H9 or C6 and secondary antibody bound scant (2+) to moderate (3+) gold (Fig. 1a and 1b). In addition, distribution of the flocculent layer on yeast-phase cells and germ tubes was unaltered by trypsin treatment. Although the electron micrographs shown in Fig. 1 represent cells grown for 2.5 h, cells at all time periods (1, 2, 4, and 20 h) as well as cells treated with either monoclonal antibody in nongerminating populations at 0, 15, and 30 min reacted similarly.

Trypsin treatment was therefore incorporated into preparation of cells for IEM as follows. Stationary-phase blastospores were washed in prewarmed PBS (37°C) and counted in a hemocytometer, and 5×10^7 cells were inoculated into 100 ml of prewarmed (37°C) GM-2 in a 125-ml Erlenmeyer flask. Germination was suppressed in control flasks either by the addition of a crude extract of morphogenic autoregulatory substance or by the inoculation of 10^8 cells per ml (10). All flasks were rotated (160 rpm) at 37°C. At 0.25, 0.5, 1, 2, 4, and 20 h, single flasks of germinating and nongerminating cells were removed, and the cells were washed in ice-cold PBS and trypsin treated as described above. After washing in cold PBS the cells were fixed in 0.05% Formalin for 15 min at 22 to 24°C and reacted with antibodies C6, H9, or ascitic fluid without antibody (as a control) as previously described (5). The cells were then reacted with goat anti-mouse immunoglobulin conjugated with 20-nm colloidal gold particles (E-Y Laboratories, Inc., San Mateo, Calif.) and prepared for electron microscopy as described in the accompanying paper (5). A minimum of 10 thin sections per bullet was examined from duplicate experiments, and an unbiased observer evaluated all sections as they were viewed in the microscope.

Antigens which reacted with monoclonal antibodies were associated with a flocculent layer on the outer surface of both yeast-phase cells and hyphae. Table 1 summarizes the results of the antigenic variability studies of *C. albicans* 105 in germinating and nongerminating cells. Seventy-five percent of both germinating and nongerminating cells, when reacted with H9 after 0 h (washed cells used to inoculate GM-2) or 0.25 h of incubation, bound 2+ to 3+ colloidal gold (Fig. 2a), but a few cells were negative or heavily labeled. At 30 min into germination most cells bound gold scantily (2+), and less than 10% of the cells were negative. Germ tubes, first apparent at 1 h, bound 2+ gold, but mother cells were unlabeled (Fig. 2b). Nongerminating cells observed at 1 h varied in the amount of H9-specific antigen expressed; 75% of the cells were either 3+ or 4+ labeled, whereas others were only 1+ or 2+ labeled. By 2 h all germinating mother cells were unlabeled, and all germ tubes were labeled 2+ to 3+, most heavily at the apices (Fig. 2c), whereas 95% of nongerminating cells were 2+ or 3+ labeled. At 4 h approximately 1% of the mother cells had reexpressed a small amount (1+ to 2+) of surface antigen (Fig. 2d). Nongerminating cells remained essentially unchanged. Thorough searching of sections was required to discern mother cells within hyphal masses at 20 h, but in all cases where this was accomplished mother cells had reexpressed a large quantity

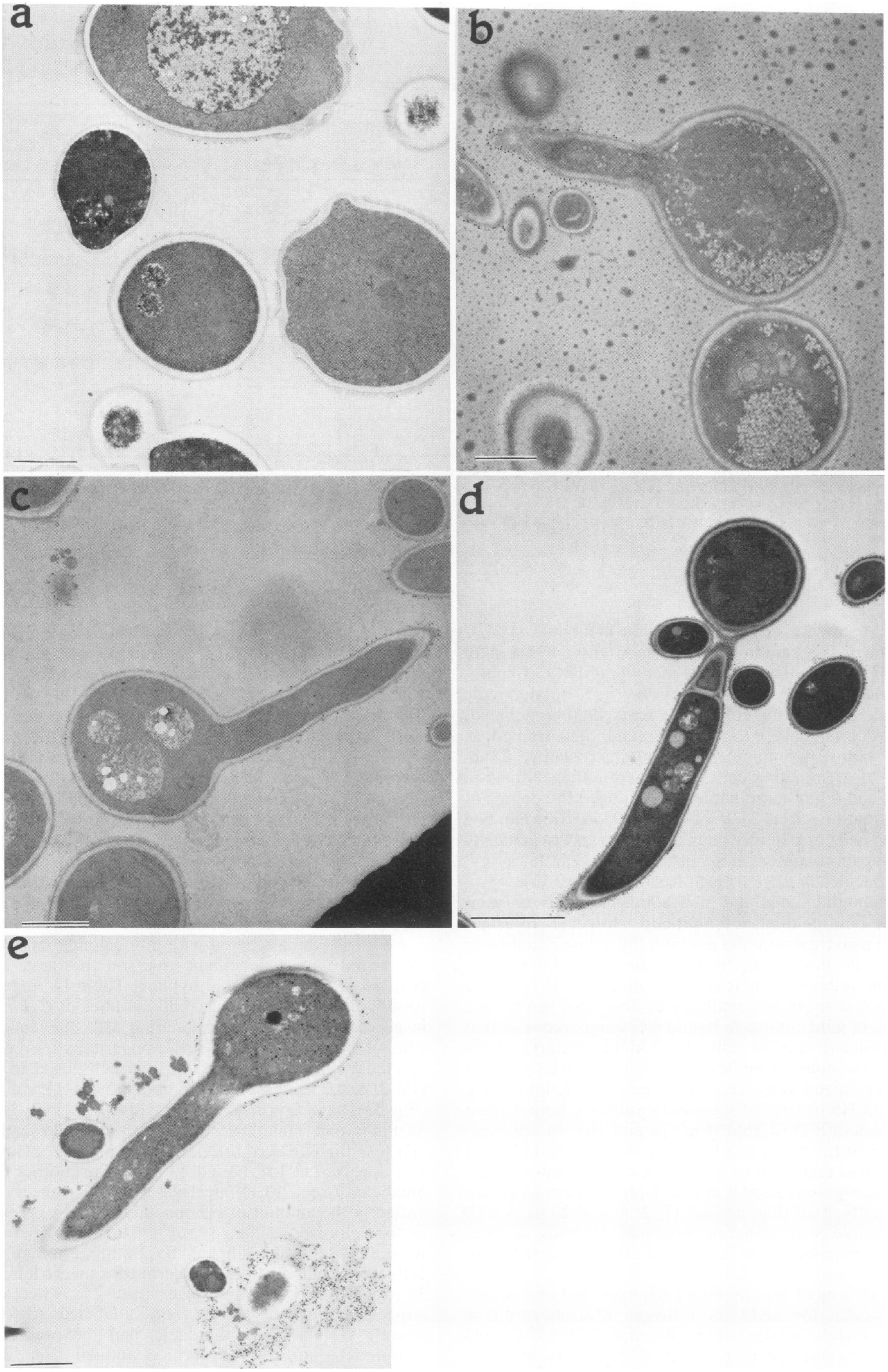

FIG. 2. Variable expression of antigenic determinants specific for monoclonal antibody H9 during morphogenesis of *C. albicans*. Shown are (a) yeast cells suppressed from germinating at 0 h, (b) cells at 1 h of germination, cells at 2 and 4 h of germination (d and e, respectively), and (e) cells at 20 h of germination. Bars, 1 μm in a, b, c, and e and 2 μm in d.

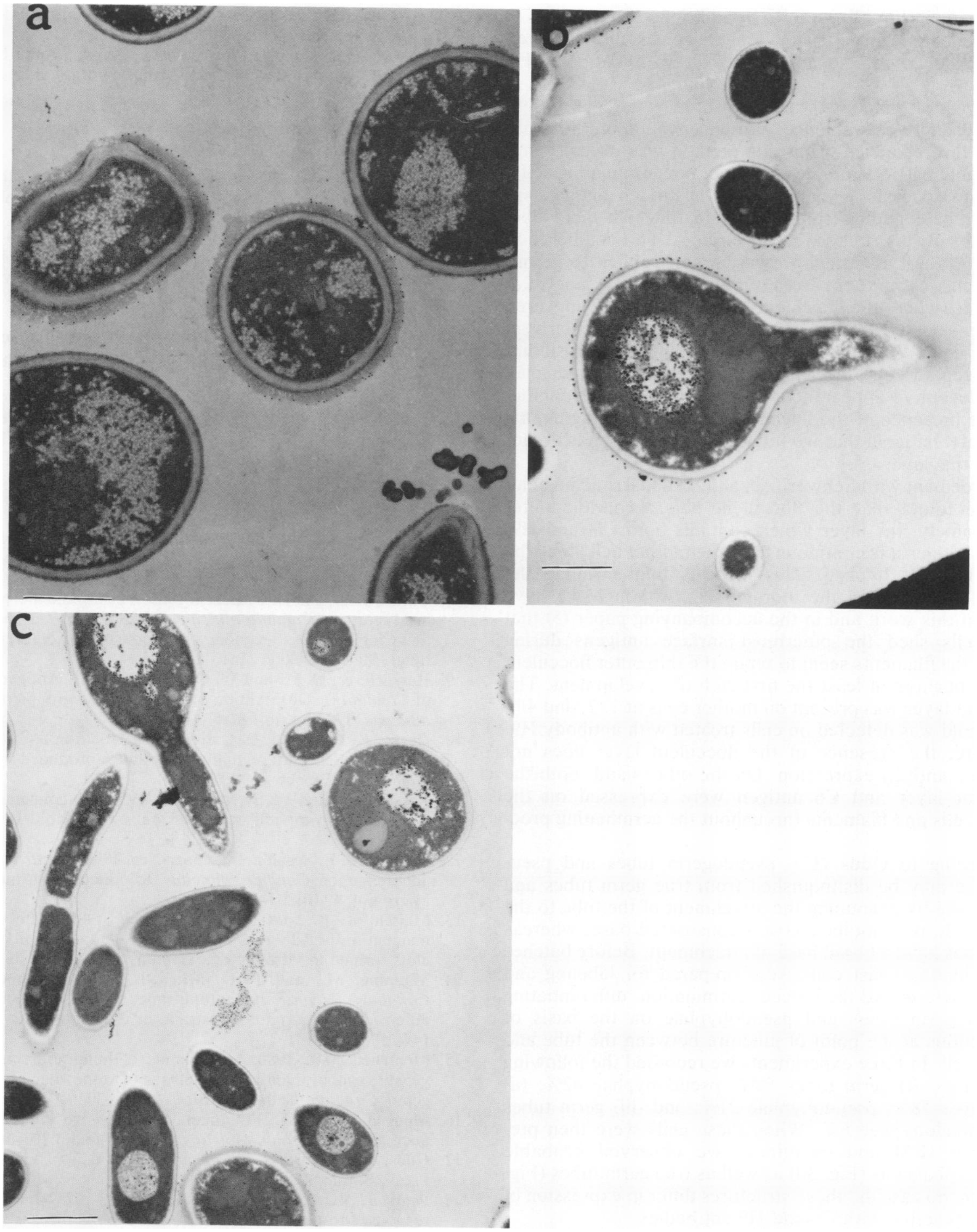

FIG. 3. Variability in expression of the antigenic determinant for monoclonal antibody C6 during morphogenesis of *C. albicans*. Shown are (a) yeast cells suppressed from germination at 0.25 h, (b) cells at 1 h of germination, and (c) cells at 4 h of germination. Bars, 1 μm in a and b and 2 μm in c.

(4+) of AgH9, and germ tubes retained 2+ label (Fig. 2e). Although the germ tube in Fig. 2e appears to be shorter than expected after 20 h of growth, this undoubtedly represents an angular cut through the proximal portion of the tube.

Cells used to inoculate GM-2 (0 h) bound 1+ to 2+ gold after reacting with C6 antibody. Yeast cells suppressed from germinating varied in AgC6 expression (rare to moderate amounts bound) during all time periods tested (Fig. 3a). Antigen expression was scanty (2+) to moderate (3+) on cells examined at 30 min of germination. In marked contrast to results obtained with the H9 antibody, all germinating mother cells were reactive with C6 antibody at 1, 2, and 4 h of incubation (Fig. 3b and c). In addition, nongerminating cells incubated for 1 h or longer consistently reacted less intensely with C6 antibody than with antibody H9 (Table 1).

Methods to semiquantify surface antigens (i.e., 1+ to 4+) were chosen by convenience, and we encountered no problems or discrepancies when this method was used to enumerate gold particles on the surface of nongerminating cells. Except for occasional patches where surface antigen appeared to be denuding from nongerminating cells, gold label was uniformly distributed on cell surfaces. This enumeration system was not as straightforward when applied to germinating cells. Germ tubes were often more heavily labeled at the apices, where the flocculent layer consistently appeared to be most dense, than along the filament, particularly in younger cells. We cannot, however, exclude the possibility that tangential cuts through the flocculent outer layer may give a deceptive appearance of heavier labeling. Nonetheless, the presence of the flocculent layer at growing hyphal tips is interesting in that we found that growing buds do not express this layer (5).

In agreement with Schweritz et al. (21) and Tronchin et al. (26), we found that the flocculent polysaccharidic outer layer, namely, the layer which contains both C6- and H9-specific antigens, is continous from the mother cell along the germ tube and hyphae. This layer is thinner along the filament than around the mother cell. Although we have shown in this work and in the accompanying paper (5) that yeast cells shed the outermost surface antigens during growth, the filaments seem to retain the thin outer flocculent layer throughout at least the first 20 h of development. The flocculent layer was present on mother cells at 1, 2, and 4 h, but no gold was detected on cells treated with antibody H9. Therefore, the presence of the flocculent layer does not guarantee antigen expression. On the other hand, both the flocculent layer and C6 antigen were expressed on the mother cells and filaments throughout the germinating process.

According to Odds (17), pseudogerm tubes and pseudohyphae may be distinquished from true germ tubes and true hyphae by examining the attachment of the tube to the mother cell; pseudotubes have a constricted base, whereas germ tubes have a broad base of attachment. Before batches of germinating yeast cells were prepared for labeling and IEM we determined the percent germination, differentiating between germ tubes and pseudohyphae on the basis of constrictions at the point of juncture between the tube and mother cell. In three experiments we recorded the following percentages: (i) germ tubes 58%, pseudohyphae 42%; (ii) germ tubes 78%, pseudohyphae 21%; and (iii) germ tubes 91%, pseudohyphae 6%. When these cells were then prepared for IEM and examined, we observed probable pseudohyphal cells (Fig. 3d) as well as true germ tubes (Fig. 3e), yet in no case did these structures differ in expression of antigens reactive with C6 and H9 antibodies.

Our studies are provocative because they suggest that *C. albicans* can express some antigens continuously (e.g., AgC6) during hyphal development, whereas others (e.g., AgH9) may be produced periodically. The antigen complement found on candidal surfaces may be influenced by nutritional and additional environmental factors (4, 5). Confusion regarding the role of antibodies, cell-mediated immune responses, and other aspects of host defense against candidiasis (17) may relate to our inadequate understanding of control and regulation of antigen expression by this fungus.

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