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Caveats in the Investigation of Form-Specific Molecules of *Candida albicans*

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Numerous reports purporting the existence of form-specific antigens of *Candida albicans* have been published, but it is generally unclear whether antigenic variability is an acceptable alternative interpretation. In this study, we used indirect immunofluorescence and immunogold electron microscopy to determine the distribution and form specificities of two antigens during yeast and hyphal growth in several defined and complex media. The results confirmed that antigen expression varies with length of incubation, nutrition, and serotype and indicate that the form specificities of antigens may be misinterpreted when conclusions are based exclusively on indirect immunofluorescence and extraction procedures. We therefore suggest that investigations be designed to include serotype A and B isolates grown in both complex and chemically defined media and that agglutination, immunofluorescence, or enzyme-linked immunosorbent assays on whole cells or cell extracts be used as presumptive tests. Confirmation of form-specific antigens should be done by appropriate immunoelectron microscopic evaluation.

Candida albicans, a commensal dimorphic yeast, has emerged as a serious opportunistic pathogen in the immunocompromised host. The organism is capable of growing as a budding yeast, septate hyphae, or pseudohyphae (22). Surface antigens, most of which remain undefined, make initial contact with host immune mechanisms and play an important role in antigen presentation. The list of possible virulence factors associated with the cell wall has grown to include adhesins, acid proteases, the ability to produce germ tubes, hydrophobicity, expression of complement receptors, and antigenic variability (3–5, 10–14, 20, 23, 26).

As a commensal, *C. albicans* is found as a yeast on mucocutaneous surfaces. However, yeast and hyphal forms of the organism are found in candidal lesions. Agglutinating antibodies which react with yeast cells are found in most healthy individuals (34) and confound the serodiagnosis of disseminated infections. Consequently, the search continues for specific markers which distinguish superficial infections from life-threatening diseases. Because hyphae are considered the hallmarks of tissue invasion, the discovery of antigens expressed exclusively by this form would be useful in the diagnosis of invasive disease and in the development of vaccines for populations at risk. In the last half-decade, reports of the discovery and partial characterization of form-specific molecules have been published (1, 8, 25, 28–30, 33). In these studies, yeast cells or hyphae were often examined at single time points during growth, and antigen expression was frequently defined either on the basis of indirect immunofluorescence assays (IFA) with polyclonal or monoclonal antisera or by enzyme-linked immunosorbent assay titers. Only in rare instances was antigen expression examined in more than a single growth medium or at more than a single time point in either growth form. We and others have shown that surface antigen expression in vivo or in vitro in both yeast and hyphal forms of *C. albicans* is a dynamic process and is subject to changes in growth conditions (3, 5, 6, 9, 24, 26, 32). Therefore, we present evidence to establish the necessity of rigorously examining *C. albicans* cells in yeast form and during morphogenesis in a variety of media and in temporal studies to verify stringent form specificity of antigen expression before conclusions are made. We also suggest minimum requirements for differentiating conditional and stringent antigenic form specificities on the basis of our own experiences with two dynamically expressed surface antigens.

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MATERIALS AND METHODS

Organisms and culture conditions. Two clinical isolates of *C. albicans* from disseminated candidiasis were used in these studies. Isolate Ca105, a serotype B strain, was described previously (6) and is designated Ca105(B). The second isolate was received from the Clinical Microbiology Laboratories of the University of Washington, Seattle. It was a serotype A strain obtained from an endophthalmic lesion in an immunosuppressed patient and is designated CaOS(A). Both strains were maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) with minimal subculturing. For the experiments, the *Candida* strains were maintained in yeast form by growth in glucose-salts-biotin medium (GSB) (29) at 25°C for 48 h, either in glucose (2%)–yeast extract (0.3%)–peptone (1%) broth (GYEPB) at 25 or 37°C for 24 or 48 h or in synthetic amino acid medium for fungi (SAAMF; American Biorganics, North Tonewanda, N.Y.). Washed yeast cells grown in either GYEPB or GSB medium were allowed to germinate in 10% calf serum (GIBCO Laboratories, Grand Island, N.Y.), a medium described by Lee et al. (L medium [18]), modified L medium (ML medium [31]), or a defined germination medium (GM-2 [15]).

Production of monoclonal antibodies. Two monoclonal antibodies (C6 and H9) used in this study have been described and characterized as agglutinating immunoglobulin M antibodies (5, 6).

IFA. Ca105(B) and CaOS(A) were grown in GSB at 22 to 24°C with rotation (200 rpm) for 48 h, in GYEPB at 37°C with rotation for 24 h, in GYEPB at 22 to 24°C with rotation for 48 h, or in SAAMF at 37°C with rotation for 24 h. GSB-grown yeast cells were washed twice in distilled water, and 2×10^8

cells were inoculated into 100 ml of fresh, prewarmed (37°C) ML or L medium to allow germination. GYEPB-grown yeast cells (37°C) were washed, and 2×10^8 cells were inoculated into either 100 ml of prewarmed 10% calf serum in distilled water or 100 ml of prewarmed GM-2 for germination. Washed SAAMF-grown yeast cells (10^8) were inoculated into 100 ml of fresh prewarmed SAAMF. All cultures were incubated at 37°C and 200 rpm. At 0.0 h (when cells used for inoculum were washed), 0.25, 0.5, 1, 2, 3, 4, and 24 h, samples of cells were removed, washed three times in ice-cold distilled water, placed onto glass slides, and allowed to air dry. At 24 and 48 h, yeast cells grown in GYEPB at 22 to 24°C were also washed, placed onto slides for staining, and air dried. The slides were gently heat fixed, and IFAs were done by methods previously described (3), except that undiluted hybridoma cell supernatant fluid (agglutinin titer, 1:8) containing either H9 or C6 was used as the primary antibody. Two fluorescein isothiocyanate-conjugated goat anti-mouse antibody preparations produced by different manufacturers (Sigma Diagnostics, Inc., St. Louis, Mo. and Organon Teknika, Malvern, Pa.) were compared. All stains were done in quadruplicate on at least two separate occasions per experimental observation. The stained slides were mounted in Aquamount (Lerner Laboratories, Pittsburgh, Pa.), examined with a fluorescence microscope (Ortholux II; Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, N.J.), and graded for intensity and distribution of fluorescence as previously described (3). Although *C. albicans* yeast cells and hyphae are universally autofluorescent, we refer to cells which lack specific immunofluorescence as nonfluorescent or negative for the purpose of clarity. Positive and negative controls were made with *C. albicans* 9938, which reacts strongly with both H9 and C6, and with *Saccharomyces cerevisiae,* which is known to be nonreactive with either antibody (3, 4).

IEM. Methods of pre- and postembedment immunogold electron microscopy (IEM) have been previously described (4–6). Yeast cells incubated at room temperature in GYEPB and GSB media for 24 and 48 h and germinating cells growing in 10% serum and L and ML media were examined by pre- and postembedment IEM for the expression of antigens which react specifically with H9 and C6 (AgH9 and AgC6, respectively) at 0.0, 0.25, 0.5, 1, 2, 3, 4, and 24 h of growth. The sources of the primary and secondary antibodies used in pre- and postembedment IEM were undiluted hybridoma cell supernatant fluid (agglutinin titers, 1:8) and goat anti-mouse antibody conjugated to 10-nm gold particles (E-Y Laboratories, San Mateo, Calif.), respectively. The expression of AgC6 and AgH9 by Ca105(B) in GM-2 has been previously reported (5).

RESULTS

IFA with H9. Regardless of growth medium or *Candida* strain, yeast cells which were positive for AgH9 expression became nonfluorescent by 0.5 h, under germinative conditions. By 4 h, the antigens reappeared on germ tubes in an often mottled and nonuniform distribution, and most hyphae retained at least a weak fluorescence over the next 20 h. The distribution of AgH9 on CaOS(A) cells maintained in yeast form in GYEPB at 37 or 22 to 25°C varied (from 1 to 4+, where 1+ is very weak fluorescence and 4+ is very strong fluorescence), but over two-thirds of the cells were moderately or strongly fluorescent. CaOS(A) cells maintained in yeast form in SAAMF for 6 and 24 h were weakly fluorescent (1 to 2+). In two independent experiments, over 90% of cells grown in GSB at 22 to 24°C were negative by IFA, and the rest were weakly or moderately fluorescent (2 or 3+) at 24 and 48 h. However, in two other experiments, all cells were negative by IFA irrespective of which secondary antibody was used.

When GSB-grown yeast cells were allowed to germinate in ML medium, mother cells and nongerminating blastoconidia were negative, but the distal portions of germ tubes were strongly fluorescent at 2 to 4 h, and the entire length of some germ tubes fluoresced (Fig. 1a and b). During germination in GM-2, the distal portions of the germ tubes were fluorescent, but the fluorescence was weak and patchy by 4 and 24 h. When yeast cells were permitted to germinate in 10% serum, the fluorescence was strong and uniform on germ tubes by 1 to 2 h and mother cells were consistently negative (Fig. 1c and d). Observations based on these results could have led erroneously to the conclusion that H9 antigen is hypha specific.

Regardless of growth medium or length of incubation, most Ca105(B) yeast cells were negative. Between 10 and 30% of the blastoconidia grown in GYEPB or GSB medium had fluorescence intensities of 2 to 3+, while almost half the cells maintained as yeast in SAAMF had fluorescence intensities of 1 to 2+ at 2 and 24 h.

IFA with C6. With few exceptions, >90% of blastoconidia and mother cells of CaOS(A) and Ca105(B) were strongly fluorescent (3 to 4+) and germ tubes were negative at all times tested, regardless of growth medium or growth conditions (Fig. 1e through h). The only exception to these observations was that approximately 50% of CaOS(A) yeast cells had fluorescence intensities of 3+ when grown in GSB medium at 22 to 25°C for 48 h and in L and ML media at 0.25 and 0.5 h. The remaining cells were negative. The tips of emerging germ tubes of CaOS(A) and Ca105(B) were fluorescent during early germination in GM-2 (Fig. 1i), but the fluorescence disappeared with hyphal elongation. Regardless of germination medium, a small percentage (<10%) of mother cells were negative at 3 and 4 h after germination. These data tend to indicate that AgC6 is a yeast-form-specific molecule.

IEM. The intra- and extracellular distributions of AgH9 and AgC6 have been reported previously for Ca105(B) grown in GM-2 (6). When CaOS(A) blastoconidia grown in GYEPB at 22 to 24 or 37°C were reacted with AgC6, >90% of the cells showed a dense bilayered distribution of AgC6, whereas <50% of yeast cells grown at 22 to 24°C in GSB medium were labeled. Labeling was confined to patchy areas of a flocculent layer that appeared to be sloughing from the surfaces of the cells (Fig. 2a). When AgC6 distribution on CaOS(A) cells was examined during germination in L and LM media, the labeling was very similar to that seen during growth in GM-2 and 10% serum. Antigen was distributed in a bilayer on the mother cell, and moderate amounts of AgC6 were located in deep cell wall layers along the entire length of the hyphae (Fig. 2b and c). In <10% of the cells, AgC6 was distributed almost exclusively in the internal layers of the mother cells and germ tubes in moderate amounts, with rare to scant label appearing on the surface. These observations demonstrate that AgC6 is not expressed exclusively by yeast-form cells.

DISCUSSION

The investigation of *C. albicans* form-specific antigens may lead to a better understanding of host-candida interactions, the development of diagnostic tests for disseminated

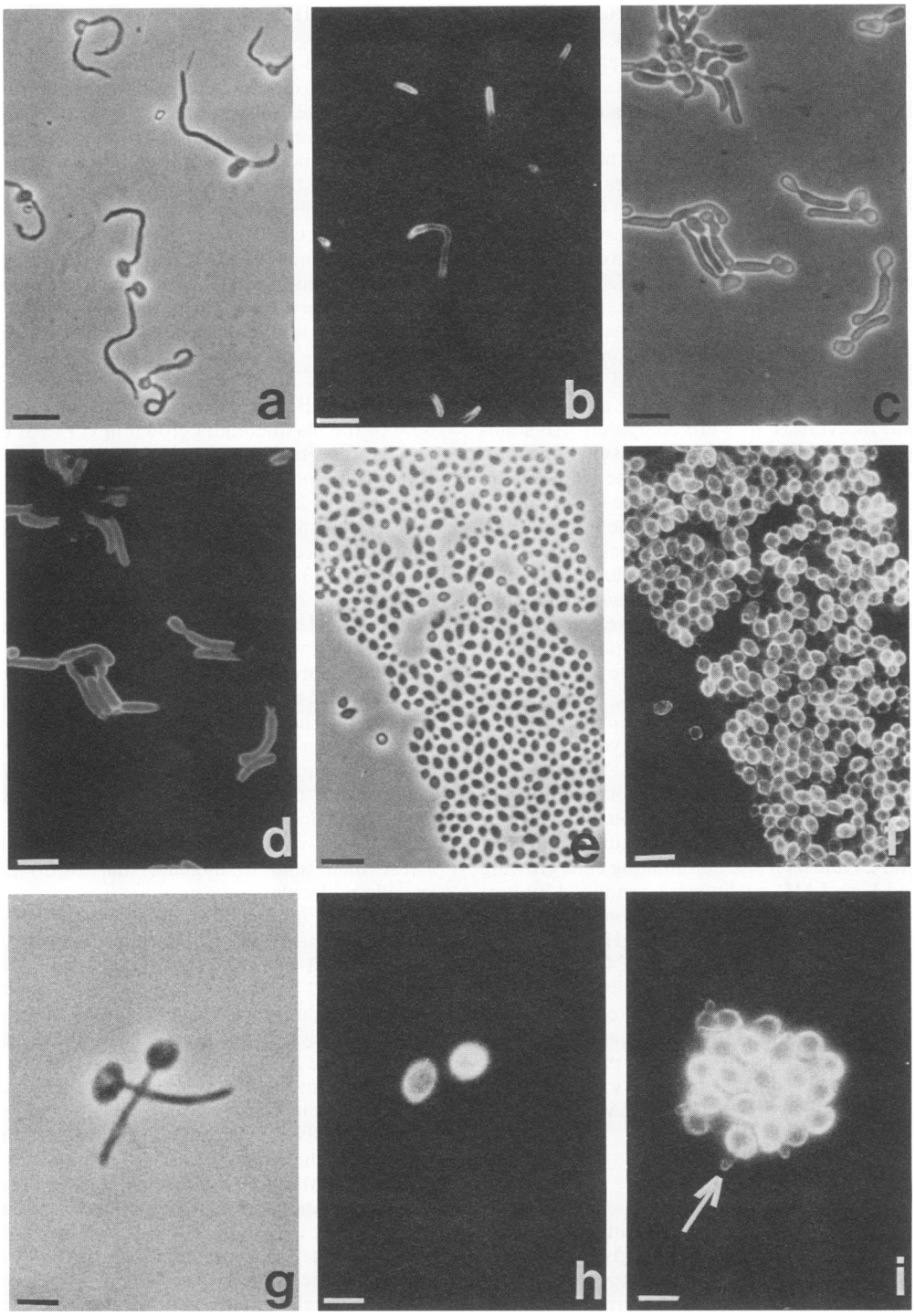

FIG. 1. Bright-field and immunofluorescence photomicrographs of *C. albicans* reacted with monoclonal antibodies H9 and C6. (a and b) H9-reacted CaOS(A) cells germinated in L medium for 4 h at 37°C. The distal portions of the germ tubes and occasionally the entire germ tube are strongly fluorescent. (c and d) H9-reacted CaOS(A) yeast cell germinating for 2 h in 10% serum at 37°C. Fluorescence is uniformly strong on germ tubes. Mother cells are nonfluorescent. (e and f) CaOS(A) yeast cells grown in GYEPB at 37°C for 24 h and reacted with C6. Ninety percent of the yeast cells are strongly fluorescent. (g and h) C6-reacted CaOS(A) at 2 h of germination in L medium. Mother cells mostly have fluorescence intensities of 4+. Germ tubes are uniformly nonfluorescent. (i) C6-specific fluorescence of emerging germ tubes (arrows) of Ca105(B) at 0.5 h in GM-2. Apical fluorescence disappeared by 1 h. Bars = 25 μm (a through f), 8 μm (g and h), and 10 μm (i).

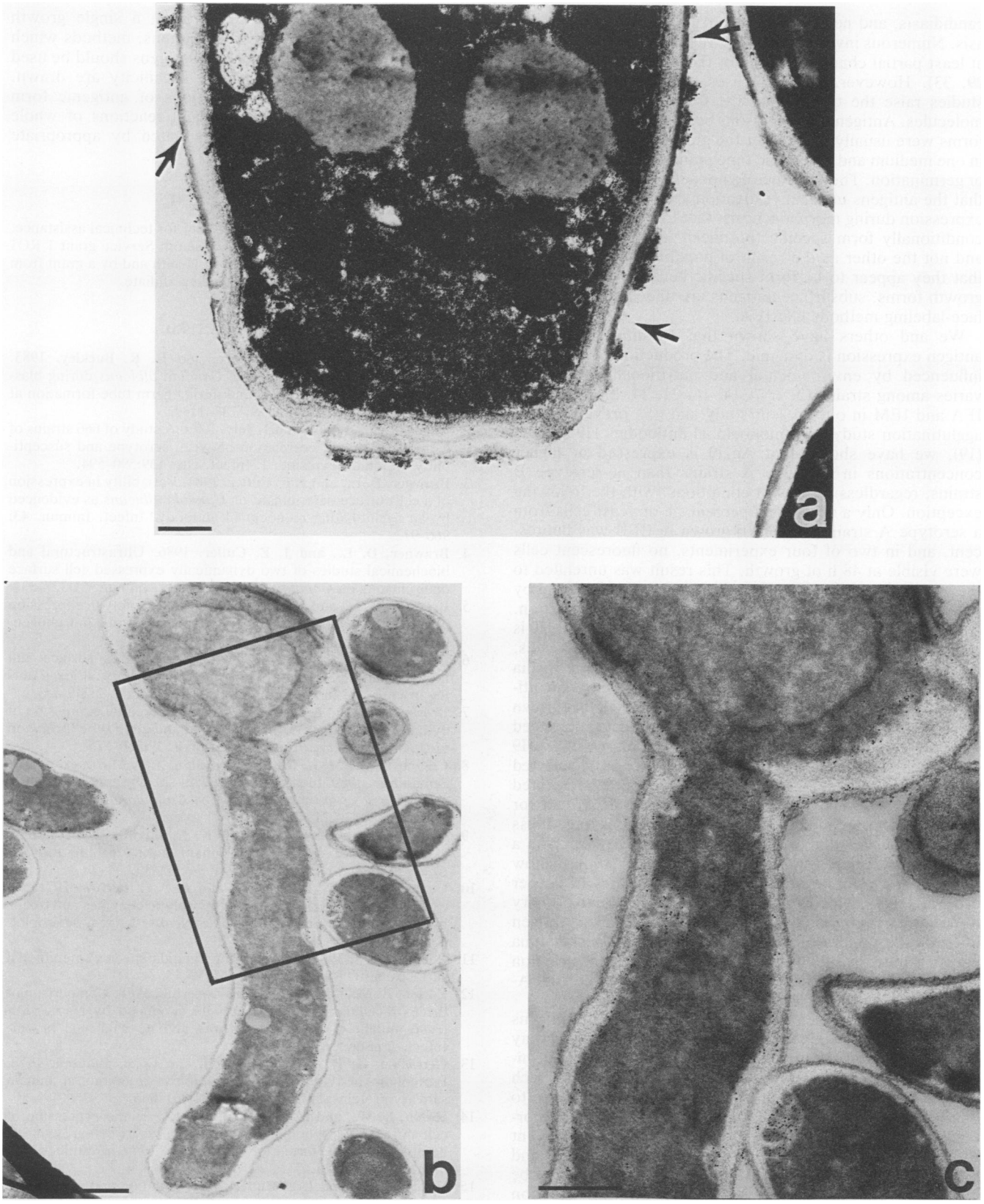

FIG. 2. Immunoelectron photomicrographs of *C. albicans* reacted with C6 or H9 and immunogold-labeled secondary antibody. (a) CaOS(A) grown for 48 h in GSB and reacted with H9. The outer flocculent area is sloughing from the cell surface (arrow). (b and c [enlargement of boxed area in panel b]) CaOS(A) was germinated in ML medium for 3 h and reacted with C6 antibody. AgC6 is labeled on the external surface of the mother cell and internally in the mother cell and hyphae. Bars = 1.0 μm.

candidiasis, and new approaches to the therapy of candidiasis. Numerous investigators have reported the isolation and at least partial characterization of these antigens (8, 25, 28, 29, 33). However, in most cases, the limitations of the studies raise the question of the form specificities of the molecules. Antigenic comparisons between yeast and hyphal forms were usually made after the growth of a single isolate in one medium and at a single time point during yeast growth or germination. These studies do not address the possibilities that the antigens under investigation are subject to variable expression during morphogenesis, that they could be merely conditionally form specific (produced in one growth form and not the other as the result of conditions of growth), or that they appear to be form specific because in one of the growth forms, subsurface antigens are undetectable by surface-labeling methods like IFA.

We and others have shown that *C. albicans* surface antigen expression is dynamic. The production of antigen is influenced by environmental and nutritional factors and varies among strains (2, 3, 7, 14, 16, 21, 24, 27). By using IFA and IEM in our present study and in a previous yeast agglutination study with monoclonal antibodies H9 and C6 (19), we have shown that AgH9 is expressed at higher concentrations in serotype A strains than in serotype B strains, regardless of growth conditions, with the following exception. Only a very small percentage of yeast cells from a serotype A strain [CaOS(A)] grown in GSB was fluorescent, and in two of four experiments, no fluorescent cells were visible at 48 h of growth. This result was unrelated to the lot of secondary antibody used but may be explained by antigen shedding (4) which precluded IFA antigen detection. Despite the lack of fluorescence, IEM revealed that AgH9 is produced in low concentrations on GSB-grown yeast cells. In addition, growth in other complex and synthetic media confirmed the ability of these cells to produce large quantities of AgH9 during yeast-phase growth, and GSB-grown yeast cells which were allowed to germinate produced detectable AgH9 on germ tubes. If observations of AgH9 distribution had been restricted to IFA detection of selected GSB-grown yeast cells, AgH9 would have been interpreted erroneously to be hypha specific. Likewise, IFA data for AgC6 distribution on yeast-form and hyphal cells, regardless of nutritional or temporal factors, indicate that AgC6 is a yeast-form-specific antigen. However, IEM studies show that AgC6 is expressed in substantial quantities in deeper cell wall layers, which are apparently inaccessible to primary or secondary antibodies or both in intact cells. Antigen buried in deep cell wall layers or beneath the plasmalemma may not necessarily be solubilized by routine extraction methods. Alternatively, as others have shown (17), IFA-negative cells may contain extractable antigen.

In conclusion, the expression and distribution of antigens on hyphae and blastoconidia are strain dependent and may vary with growth medium and length of incubation. Germination is a continuous process or sequence of events which culminates in the formation of hyphae. The time chosen to observe events relating to antigen expression during morphogenesis is critical. Therefore, to confirm the stringent form specificities of *C. albicans* antigens, we recommend that more than a single strain and serotype of *C. albicans* be examined and that the expression of the antigen in question be determined from yeast-form cells grown in complex and chemically defined media and from hyphal cells at several periods during early and late germination. In addition, regardless of whether observations are confined to the investigation of conditionally expressed antigens (i.e., antigens produced by a single strain or in a single growth medium) or stringently expressed antigens, methods which detect subsurface as well as surface antigens should be used before conclusions regarding form specificity are drawn. Therefore, presumptive determinations of antigenic form specificity by standard immunological reactions of whole cells or extracts should be substantiated by appropriate methods, such as IEM.

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