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# Variable Expression of a Surface Determinant during Proliferation of *Candida albicans*

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**The surface expression of an antigenic determinant that is present in the cell wall of *Candida albicans* was investigated with monoclonal antibody 24 (MAb24), an immunoglobulin M MAb. The proportion of the cell population that expressed the epitope under different growing conditions was determined by indirect immunofluorescence microscopy. More than 90% of stationary-phase yeast cells of strain B311 grown at 28°C expressed the antigen. Less than 50% of yeast cells grown exponentially at 28°C or either growing or stationary-phase yeast cells cultivated at 37°C expressed the epitope. Germ tubes, which were induced at 37°C from stationary-phase yeast cells grown at 28°C, expressed the determinant on the parent yeast but not the hyphal portion of the germ tube. The change in antigen expression by stationary-phase cells grown at 28°C, when they resumed growth by bud formation, suggested that antigen expression was lost by cells in the inoculum prior to the first cell division. By using the same assay, strong positive reactions were observed in stationary-phase cultures of other isolates of *C. albicans*, *C. guilliermondii*, *C. stellatoidea*, and *C. tropicalis*, but not with isolates of *C. krusei*, *C. parapsilosis*, or *Torulopsis glabrata*. The identification of the antigenic determinant as a carbohydrate was based on three observations: (i) interaction with a mannan preparation from the same organism, (ii) sensitivity of the antigen to periodate but not proteases, and (iii) coincidence of the migration of antigen during electrophoresis with material which stained intensely with carbohydrate but not with protein reagents. These observations suggest that the expression of the antigenic determinant of MAb24 is dependent on the growth conditions, growth state, and morphology of the cell and that the topography of the cell surface is dynamic.**

The cell wall of *Candida albicans* is the site of the initial interaction between the organism and its environment. In addition, the wall maintains the structural shapes which characterize each growth form of the dimorphic fungus and is part of the permeability barrier of the cell. As the outermost part of the cell wall, the surface, in particular, is of interest in the mediation of adherence in colonization and invasion of tissue and presentation of antigens to the host. However, the surface moieties and their antigenic determinants remain generally undefined.

By using concanavalin A, a lectin with affinity for mannose polymers, results of cytochemical, ultrastructural, and agglutination studies have shown that mannose-containing constituents are present at the surface (5, 31, 33). The surface exposure of protein constituents has been demonstrated by the accessibility of these substrates to protease (5, 18, 21, 27, 30). Results of studies with polyclonal antisera have shown that antigenic determinants may not be expressed in all strains or under all conditions in a single strain (12, 24–26, 29). However, in these studies the individual constituents containing mannose or protein which are exposed were not defined and antigenic changes were not identified. The definition of the surface constituents and their antigenic determinants is important to the understanding of the structure-function relationships of the cell wall in host-parasite interactions and in the maintenance of structural forms and permeability. In several studies surface structures have been specifically identified. One specific constituent, the enzyme acid phosphatase, is present at both the surface and near the plasma membrane (32). Monoclonal antibodies (MAbs) have been used to identify other surface antigens, most of which are predominantly polysaccharide in nature (2, 3, 14, 23). The expression of epitopes identified by three of these MAbs varied with the morphology of the cell or the age of the culture (2–4, 14).

In the present study, a MAb against a cell surface component was used to investigate the surface expression of the epitope during cellular proliferation. In this case indirect immunofluorescence was used more extensively than in other studies to provide a quantitative measurement of the proportion of the *C. albicans* population that expresses the surface antigen. The chemical nature of the epitope and the prevalence of surface expression of the determinant by other strains and species were examined.

## MATERIALS AND METHODS

**Organisms and culture conditions.** *C. albicans* B311 was used in these experiments, unless otherwise noted. The other organisms used were six isolates of *C. albicans*, one isolate of *C. parapsilosis*, four isolates of *Torulopsis glabrata* (obtained from B. H. Cooper, Baylor University Medical Center, Dallas, Tex.), three isolates of *C. guilliermondii*, two isolates of *C. krusei*, two isolates of *C. parapsilosis* (obtained from R. L. Hopfer, M. D. Anderson Hospital and Tumor Institute, Houston, Tex.), and one isolate each of *C. tropicalis* and *T. glabrata* (obtained from T. G. Mitchell, Duke University Medical Center, Durham, N.C.). *Saccharomyces cerevisiae* D139-11B (*MATa leu2-3,112 his3-11 ade1 trp1 can1*) was obtained from D. B. Kaback (The University of Medicine & Dentistry of New Jersey, Newark, N.J.); and strains C276 (*MATa*/*MATα*), C276-4A (*MATa*), and C276-4B (*MATα*) were obtained from J. R. Pringle (University of Michigan, Ann Arbor, Mich.). Strains were maintained on yeast extract-peptone-agar. Cultures were grown either in

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the minimal medium supplemented with amino acids described by Lee et al. (19) or in YNBG, which contained yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Mich.). Both media contained 2% glucose. YNBG was used for the growth of cultures from which cell wall extracts were obtained and for growth to the stationary phase of the strains that were used to examine the reactivity of different species with the MAb. All cultures were grown in a gyratory incubator shaker at 180 to 200 rpm at 28 or 37°C. For growth resumption experiments, stationary-phase yeast cells of *C. albicans* B311 grown in Lee medium were centrifuged and suspended at $1 \times 10^7$ to $5 \times 10^7$ cells per ml in fresh medium at 28°C for yeast growth or at 37°C for the formation of germ tubes (8). For yeast growth at 37°C, the pH of the medium was adjusted to 4 to 4.5 from pH 7.4 (22). Exponential-phase cultures were obtained by diluting an exponential-phase culture into fresh medium at $4 \times 10^4$ to $10 \times 10^4$ cells per ml. Cultures were examined after seven to eight population doublings and after at least two population doublings before the stationary phase.

**Preparation of cell wall extracts and antigen.** Cells were broken by vortexing with glass beads in cold distilled water, and the cell walls that were obtained by centrifugation were extensively washed in cold water, as described previously (9). Soluble material was extracted by boiling for 2 to 4 min in buffer containing sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (9). Insoluble material was removed by centrifugation at $3,000 \times g$ for 10 min. The soluble material was frozen and lyophilized. The SDS was removed by extraction with solvent containing acetone-triethylamine-acetic acid-water (85:5:5:5, by volume) (15). After the material was dried, the residue was suspended in 0.1 N NaOH and immediately neutralized with $H_3PO_4$. The protein concentration was determined by the method described by Bradford (1).

Mannan was obtained from E. Reiss (Centers for Disease Control, Atlanta, Ga.).

**Immunization.** Adult male BALB/c mice received subcutaneous injections of cell wall extract containing 100 μg of protein in 0.02 ml of 0.01 M phosphate (pH 7.4)–0.15 M NaCl (phosphate-buffered saline [PBS]), which was emulsified with an equal volume of complete Freund adjuvant. The same quantity of extract without adjuvant was given intraperitoneally 3 weeks later. This procedure was repeated twice at 2-week intervals. Ten days after the fourth immunization, samples of blood were obtained and the titer of serum antibody to the immunizing antigen was determined by enzyme-linked immunosorbent assay (ELISA). The mouse that showed the highest titer received 50 μg of extract protein 3 days before it was sacrificed.

An adult male New Zealand White rabbit received intravenous injections of cell wall extract containing 1 mg of protein in 0.5 to 1 ml of PBS three times weekly for 4 weeks. The titer of the serum in an ELISA was 1:25,000.

**Production of hybridomas.** Cell fusion and selection of hybrids was performed essentially as described by Dippold et al. (10), with some modifications. Mouse plasmacytoma X63/Ag8.653 was grown in vitro in high glucose formulation Dulbecco minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% horse serum (GIBCO)–20 mM sodium bicarbonate–18 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer–1 mM sodium pyruvate–50 μM 2-mercaptoethanol–1% nonessential amino acids (Flow Laboratories, Inc., McLean, Va.)–1% antibiotic-antimycotic solution (GIBCO). For the selection of hybrid cells following fusion, this medium was supplemented with 100 μM hypoxanthine–15 μM thymidine–0.4 μM aminopterin (HAT medium). Subcloning of hybridomas was performed in medium from which the aminopterin was omitted. Polyethylene glycol (5 g; molecular weight, 4,000; Merck and Co., Inc., Rahway, N.J.) was melted for 30 min in an autoclave (0.5 kG/cm$^2$). Hot (50°C) sterile Dulbecco PBS (7 ml) containing 15% dimethyl sulfoxide was added to the melted polyethylene glycol, and the mixture was agitated until a clear viscous solution was obtained. Spleen cells from an immunized mouse were fused with X63/Ag8.653 cells at a 5:1 ratio by suspending a washed cell pellet containing both cell types in 0.2 ml of polyethylene glycol buffer for 2 min at 37°C. Fused cells were distributed into six 24-well plates (A/S Nunc, Kamstrup, Denmark), and 1 ml of HAT selection medium was added to each well. Ten days after fusion, samples of media from wells with growing hybridomas were screened for the production of antibody to the immunizing antigen. Cells from positive wells were immediately subcloned by limiting dilution in 96-well plates (A/S Nunc) or frozen in liquid nitrogen. Mouse peritoneal macrophage cells ($1 \times 10^5$ to $3 \times 10^5$ per well) were used as a feeder layer. MAbs were obtained either from confluent hybridoma cultures or from ascites. For the production of ascites, BALB/c mice were injected intraperitoneally with $10^7$ hybridoma cells. After 1 to 2 weeks, the ascites were drained with an 18-gauge needle.

The immunoglobulin class of the antibodies was determined by immunodiffusion with heavy-chain-specific reagents (Research Products International Corp., Mt. Prospect, Ill.).

**ELISA.** Slight modifications of a previously described ELISA (35) were used to analyze antibodies. Wells of Immulon I or II plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 5 to 10 μg of antigen for screening assays (Immunlon I) or with 8 to 60 ng of antigen for antigen sensitivity assays (Immulon II) in 100 μl of borate buffer and incubated at 4°C for 18 to 24 h. Borate buffer containing 6.18 g of boric acid and 9.54 g of sodium borate per liter was adjusted to pH 8.2 with HCl. The plate was rinsed three times with PBS and blocked with 200 μl of PBS containing either 0.5% gelatin or 1% bovine serum albumin (PBSB) per well for 2 to 16 h at 4°C. The plates were washed three times in PBS with 0.1% Tween 20 (PBST). Hybridoma culture supernatants or ascites fluid were diluted in PBSB and added to the appropriate wells, and the plate was incubated at 37°C for 3 h in a humid environment. The plates were rinsed five times with PBST, and 100 μl of a 1:1,500 dilution of goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (Cappel Laboratories, Cochranville, Pa.) was added to each well. The conjugated antibody was diluted in PBSB with or without 0.05% Tween 20. The plate was incubated at 37°C for 3 h and washed with PBST. Following the addition of substrate mixture containing *o*-phenylenediamine, the plate was incubated in the dark for 10 min and the reaction was stopped by the addition of 50 μl of 1 M $H_2SO_4$ to each well. The color intensity was determined empirically by eye (hybridoma screening) or read at 492 nm with an automated plate reader (Flow Laboratories). In a second assay that was used to indirectly attach antigen to the plate, rabbit anti-cell wall sera (1:1,000 dilution) or concanavalin A (2 μg) diluted in borate buffer in the first incubation was used. Prior to reaction with MAb, the antigen was diluted in PBSB and added to appropriate wells and incubated at 37°C for 2 h and washed five times with PBST. Subsequent reactions with goat anti-mouse immunoglobulin conjugated to horseradish peroxidase and with enzyme

substrate were done as described above. In the case of concanavalin A, correction was made for binding of the second antibody conjugate in the absence of MAb.

**Indirect immunofluorescence assay for detection of cell surface antigens.** The procedure for the detection of cell surface antigens by indirect immunofluorescence was modified from that described by Brawner and Cutler (2). Portions of the culture were removed and diluted with PBS. The cells were washed once with PBS and suspended in PBS at $1 \times 10^8$ to $3 \times 10^8$ cells per ml. The cell suspension (20 to 60 μl) was mixed with 50 to 150 μl of MAb (either an undiluted culture supernatant or a 1:500 dilution of ascites fluid in PBSB). The mixture was incubated for 30 to 40 min at room temperature, diluted with PBS, and washed 3 times with PBS. Rhodamine-conjugated goat anti-mouse immunoglobulin (20 to 60 μl; Cappel Laboratories) diluted 1:10 in PBSB was added and incubated for 30 to 40 min at room temperature. The mixture was diluted with PBS and washed 3 times with PBS. The cells were examined with a microscope (Zeiss Universal) equipped for epifluorescence. Fluorescence was dependent on the reaction of the cells with MAb, since no fluorescent cells were observed if cells were incubated only with the second antibody. Cells of strain B311 that reacted with antibody gave a definite unambiguous response. On a scale of −, ±, 1+, 2+, 3+, and 4+, the majority of cells were 2+ to 4+, with no cells characterized as ±. All cells that were ≥1+ were counted as positive. This criterion was used in enumerating positive cells from other strains of *C. albicans* and other *Candida* species. For strain B311, 69 to 298 cells were counted for each determination; and with one exception, 76 to 291 cells were counted for each determination with other strains and species. The observations for each cell type were compared by Student's *t* test by using $P = 0.05$ or $P = 0.01$, with the same results.

**Treatment of antigens with various reagents.** Insoluble trypsin (treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone and attached to beaded agarose), α-chymotrypsin (attached to carboxymethyl cellulose), protease from *Streptomyces griseus* (attached to carboxymethyl cellulose), and α-mannosidase were used to treat antigen preparations enzymatically. Trypsin and α-chymotrypsin were washed in 0.001 M Tris (pH 8.0). Enzyme (0.01 U/ml) and antigen (130 μg; based on protein in cell wall extracts) were incubated in 0.01 M Tris (pH 8.0; total volume, 100 μl) at 25°C with stirring for 24 h. At the end of the reaction, 0.1 μg of trypsin inhibitor was added to trypsin reactions and 0.25 μg was added to α-chymotrypsin and trypsin–α-chymotrypsin reactions. Protease was washed in 0.001 M Tris (pH 7.0), and enzyme and antigen were incubated as described above in 0.01 M Tris (pH 7.0). The insoluble enzyme was removed by centrifugation, and the supernatant was assayed. α-Mannosidase (0.01 U) was incubated with the substrate in 0.01 M sodium acetate (pH 4.8) for 24 h at 25°C. Antigen was incubated with 0.3 M sodium periodate for 18 h in the dark at 25°C in PBS. Ethylene glycol was added to 0.6 M, and the incubation was continued for 6 h. Reactions with mannan were performed similarly, except that reagent, antigen, and incubation volumes were reduced by one-half. In control experiments, incubation mixtures without antigen were added to untreated antigen during the coating of the microtiter plate for the ELISA and had no effect on the reactivity with antibody. Incubation of antigen in buffer alone did not significantly change the antigenic reactivity. The effects of the various treatments were compared by using Student's *t* test, at $p = 0.05$. Reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.).

**Polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis (PAGE) of protein was performed essentially as described by Laemmli (17). Polyacrylamide slab gels (10 or 5 to 10% linear gradient) were formed in a model SE 400 apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Subsequently, a 3% polyacrylamide stacking gel was polymerized at the top of the sample gel. Electrophoresis was performed at a constant voltage of 55 V. Slab gels were stained for protein by binding of Coomassie blue or polysaccharide by reacting them with periodic acid-Schiff reagent (11).

**Immunoblotting with histochemical stain.** After SDS-PAGE, the proteins were transferred to nitrocellulose paper essentially as described by Kyhse-Anderson (16) by using an electroblotter (semidry; SM17556; Sartorius Filters, Inc., Hayward, Calif.). Samples were transferred to nitrocellulose paper at 0.8 mA/cm$^2$ for 2 to 3 h. The filter was subsequently blocked for 6 to 15 h at room temperature or 37°C in blocking reagent containing 5% nonfat dry milk and 0.01% Antifoam A in PBS. The filter was washed five times in PBST and incubated with a 1:1,000 dilution of MAb ascites fluid in PBSB at room temperature for 2 h. The filter was subsequently washed with PBST and incubated with a 1:1,500 dilution of goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (Cappel Laboratories) in PBSB. The filter was washed as described above and rinsed in substrate buffer containing 50 mM HEPES (pH 7.4) and 150 mM NaCl. The filter was incubated with the substrates 4-chloro-1-napthol and $H_2O_2$, as described by Hawkes et al. (13). Incubations and washes employed gentle agitation.

In some experiments 3 to 10 μl of antigen diluted in borate buffer was directly applied to nitrocellulose paper. The paper was then treated with blocking reagent as described above. The filter was subsequently incubated for 2 h at room temperature with MAb in PBSB and goat anti-mouse immunoglobulin conjugated with horseradish peroxidase in PBSB. Between incubations the filter was washed with PBST, and after the last wash the filter was incubated with substrate, as described above. In controls for immunoblot experiments, a positive reaction was dependent on the presence of both antigen and MAb.

## RESULTS

**MAb24 reactivity.** In the initial screening of hybridoma reactivity with cell wall extracts and mannan, three patterns of reactivity were observed: (i) group 1 antibodies reacted strongly with both sources of antigen; (ii) group 2 antibodies reacted more strongly with the extract than the mannan fraction; and (iii) group 3 antibodies reacted exclusively, or almost so, with the extract compared with that with the mannan preparation. MAb24 belonged to group 1 and was found to belong to the immunoglobulin M class.

**Surface reactivity of intact cells.** The surface expression of the epitope was examined by an indirect immunofluorescence assay. Stationary-phase yeast cells were reacted with MAb24 and subsequently with a rhodamine-conjugated anti-mouse immunoglobulin. Microscopic examination showed that the antigenic determinant was expressed on the surface of stationary-phase cells (Fig. 1a and b). The extent of cells that expressed the antigen at the surface was 92 ± 6 ($n = 6$). There was no significant difference in expression between cells grown on Lee or YNBG medium.

**Antigen expression by *C. albicans* in different growth states and conditions.** The variability of antigen expression was examined by observing the surface reactivity of cells in

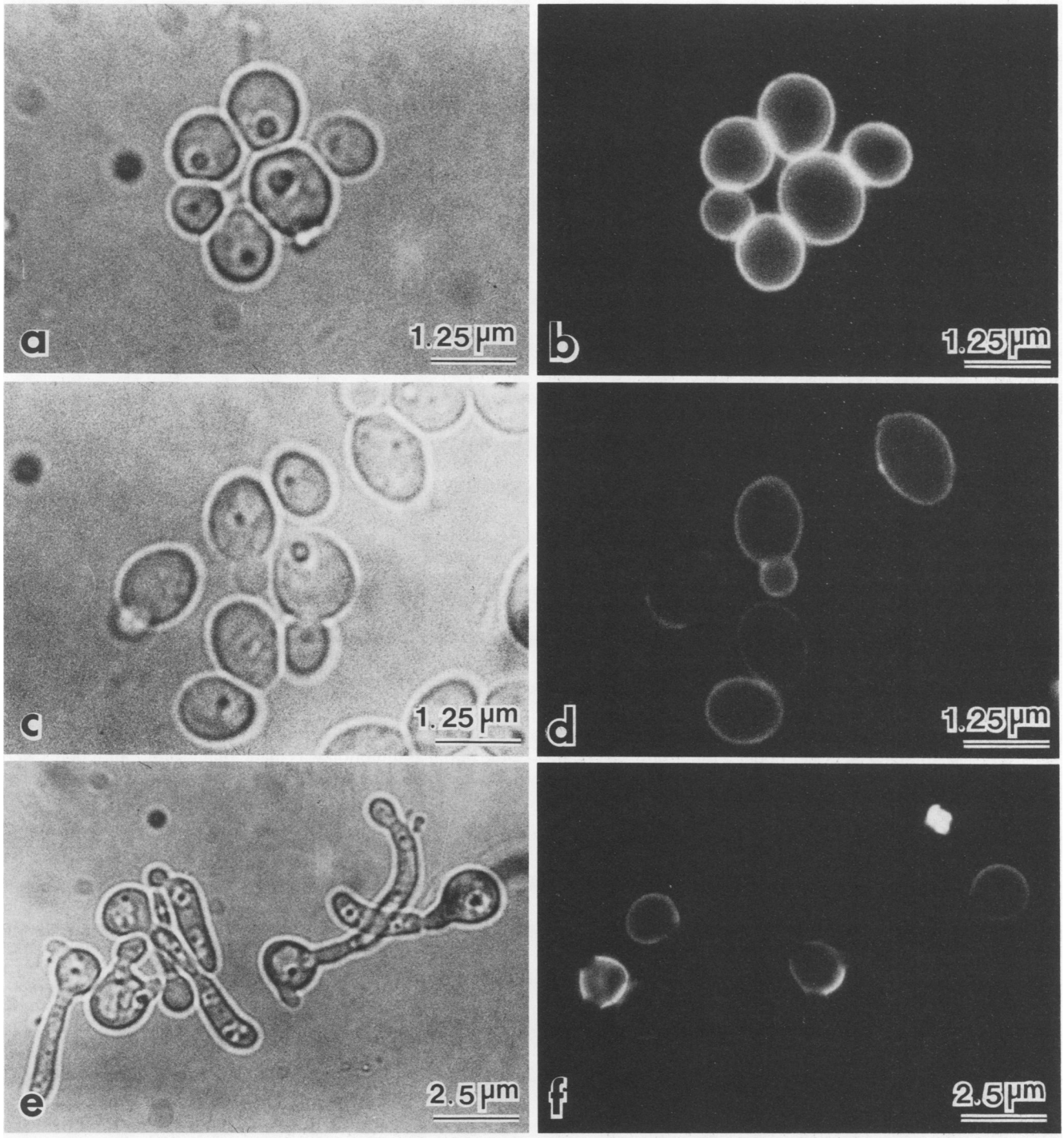


FIG. 1. Surface reactivity of intact cells with MAb24. Exponential- and stationary-phase yeast cells and germ tubes of *C. albicans* B311 were washed in PBS and incubated with MAb24, washed, and incubated with a rhodamine-conjugated goat anti-mouse immunoglobulin. The washed cells were viewed with a microscope that was equipped for epifluorescence. Each field was examined with both bright-field and fluorescence optics. Stationary-phase yeast grown at 28°C viewed with bright-field optics (a) and fluorescence optics (b); exponentially growing yeast viewed with bright-field optics (c) and fluorescence optics (d); germ tubes viewed with bright-field optics (e) and fluorescence optics (f).

different growth states with MAb24 (Table 1 and Fig. 1). Yeast cells that were growing exponentially at 28°C showed antigen expression at about half the frequency of stationary-phase cells grown at the same temperature. Yeast cells grown at 37°C and pH 4.5, whether in the stationary or exponential phase, expressed antigen at about half the frequency of the stationary-phase cells grown at 28°C. In contrast to the differences between growing and stationary-phase cells cultured at 28°C, there was no significant difference in the percentage of cells that expressed antigen between growing and nongrowing cells cultivated at 37°C. When germ tubes induced at 37°C were examined, 92% of

TABLE 1. Reaction of MAb24 with *C. albicans* in different growth states

| Growth conditions | Growth state | % Reactivity (no. of expts) |
|---|---|---|
| 28°C, pH 7.4 | Stationary-phase yeast | 92 ± 6 (6) |
| | Exponential-phase yeast | 45 ± 12 (5) |
| 37°C, pH 4.5 | Stationary-phase yeast | 39 ± 3 (3) |
| | Exponential-phase yeast | 49 ± 6 (3) |
| 37°C, pH 7.4 | Germ tube | 92 ± 5 (4)[a] |

[a] Only the parent portion of the germ tube was reactive, the elongating hypha was not fluorescent. Measurements were made 4 h after induction.

the germ tubes expressed antigen. However, antigen expression was limited to the parent portion of the structure, and there was no antigen expression on the elongating hyphal portion (Fig. 1e and f). Examination of budding cells in growing cultures showed that on individual parent-bud units, antigen could be expressed on either or both portions (Fig. 1c and d).

Stationary-phase yeast cells grown at 28°C can resume growth as yeast at either 28 or at 37°C and lowered pH. In addition, such cells can be induced to form germ tubes when placed at 37°C and pH 7.4. Because the results of the experiments described above demonstrated that there is a decline in antigen expression among yeast cells in cultures derived from inocula cells with high expression, the dynamics of antigen expression were examined during the resumption of growth. Results for one such experiment are shown in Fig. 2. After 2 h in fresh medium, the frequency of cells that expressed antigen in cultures that resumed growth in the yeast form began to decrease (Fig. 2A). The decrease was more rapid in the culture that was incubated at 37°C. When cells were suspended at 37°C under conditions that were favorable for germ tube formation, a different pattern emerged (Fig. 2B). Cells that formed germ tubes retained the high frequency of expression of the inoculum cells on the parent portion of the germ tube. The elongating hyphal portion remained negative, however, even at 8 h after induction. Cells that did not form germ tubes showed a decrease in the frequency of expression.

**Antigen expression by other isolates and species.** Additional isolates of *C. albicans* and other species were examined for surface expression of the antigen (Table 2). Cells of isolates of *C. albicans*, *C. stellatoidea*, *C. tropicalis*, and *C. guilliermondii* gave strong positive reactions. There were differences among the strains that gave positive responses. Although all isolates of *C. albicans* expressed the antigen, the extent of expression varied among the isolates. For four of seven isolates, antigen was expressed on more than 90% of the stationary-phase cells, while for only one isolate was antigen expressed on less than 25% of the yeast cells. Two isolates of *C. tropicalis* were negative for antigen expression, while a variable frequency (40 to 87%) of cells that strongly expressed antigen was observed among the positive isolates. Cells of isolates of *C. krusei*, *C. parapsilosis*, and *T. glabrata* did not show strong reactivity with MAb24, although cells of some isolates showed a very faint (±) response on as many as 39% of the cells in one case. Both diploid and haploid strains of *Saccharomyces cerevisiae* were negative for antigen expression.

**Characterization of the antigenic determinant.** Since MAb24 and other group 1 antibodies reacted strongly with the mannan preparation, which contained very little protein,

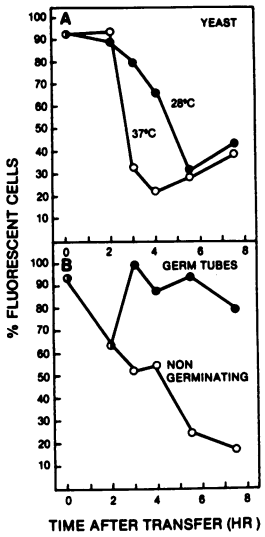


FIG. 2. Changes in surface reactivity during resumption of growth by stationary-phase cells. Yeast cells were grown to the stationary phase in Lee medium at 28°C and suspended in fresh medium to resume growth in the yeast form at 28 or 37°C and pH 4.5 (A). Germ tubes were induced by resuspension in medium at 37°C and pH 7.4 (B). At various times, washed cells from each culture were incubated with MAb24, washed, and incubated with a rhodamine-conjugated second antibody. Fluorescence was determined microscopically. Symbols for panel A: ●, Culture at 28°C; ○, culture at 37°C. Symbols for panel B: ●, Germ tubes (only the parent portion was fluorescent): ○, cells that did not form germ tubes. At early times in experiments in panel B, it was not possible to distinguish cells forming germ tubes and those that were not, and observations were recorded as total fluorescent cells.

the initial assumption was made that group 1 antibodies were directed against epitopes in the carbohydrate. This possibility was examined by two additional experiments. Both cell wall extract and mannan were separated by SDS-PAGE and analyzed by immunoblotting. The reactive material in both the extract and mannan was near the top of the gel (Fig. 3A). This area corresponds to the portion of a gel which is reactive with periodic acid-Schiff reagent following SDS-PAGE separation of a cell wall extract or mannan (Fig. 3b) (6). No reactive bands on the blotted filter were observed at positions of faster migration. The probable presence of carbohydrate in the form of mannose residues and immunoreactive material in the same moiety was examined in an additional experiment. Components of the cell wall extract

TABLE 2. Immunofluorescence assay of MAb24 with different species

| Species | No. tested | No. positive[a] | % Reactivity |
|---|---|---|---|
| *C. albicans* | 7 | 7 | 2–100 |
| *C. guilliermondii* | 3 | 3 | 51–99 |
| *C. krusei* | 2 | 0 | |
| *C. parapsilosis* | 3 | 0[b] | |
| *C. stellatoidea* | 4 | 4 | 89–100 |
| *C. tropicalis* | 6 | 4 | 40–87 |
| *T. glabrata* | 6 | 0[b,c] | |
| *S. cerevisiae* | 4 | 0 | |

[a] Strain B311 gave a strong positive reaction with MAb24. The intensity of this reaction (≥1+) was used as a standard for the other isolates.
[b] There was a very weak reactivity (±) with some cells (4 to 39%).
[c] One negative culture showed <1% reactive cells.

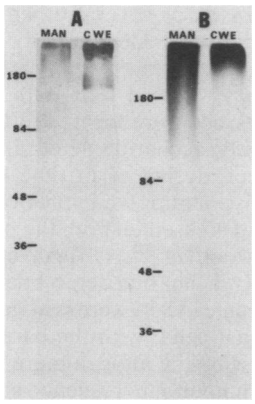


FIG. 3. Characterization of antigenic determinant. Mannan (MAN; 100 μg) and cell wall extract (CWE; 100 μg of protein) were separated by SDS-PAGE and analyzed by Western blotting or periodic acid-Schiff staining techniques. Numbers to the left of the gels are in kilodaltons. (A) Immunoblot with MAb24. (B) Periodic acid-Schiff staining.

that bound to concanavalin A were attached to a microtiter plate and separated from nonbinding fractions. The concomitant attachment of the antigenic determinant of MAb24 was analyzed by ELISA. MAb reacted with the concanavalin A-bound antigen. Therefore, it seemed probable that the cell wall constituent that contained the epitope also contained mannose residues.

The sensitivity of the antigen to conditions that degrade protein or carbohydrate was determined. The cell wall extract was incubated either with trypsin or α-chymotrypsin, or, to produce greater protein degradation, with trypsin–α-chymotrypsin or nonspecific protease. The antigenicity of the carbohydrate portion was examined by reacting the preparation with α-mannosidase, which degrades a small fraction (5%) of yeast mannan (20), or with sodium periodate. The preparations were tested by two methods. In the first method, a dot blot assay was used in which the various preparations were directly spotted onto nitrocellulose paper; and in the second method, an ELISA was used in which rabbit anti-cell wall sera was used to attach antigen to the microtiter plate. Results of the dot blot assay are shown in Fig. 4. The preparation that was treated with periodate showed a complete loss of activity, while the other treated preparations retained activity. Similarly, in the ELISA the periodate-treated preparation showed a complete loss of activity (data not shown). While only periodate-treated antigen showed a complete loss of activity, preparations treated with a combination of the specific proteases and the nonspecific protease showed some loss of activity. This decrease may have resulted from the partial destruction of the epitope or the loss of the contribution of the protein portion of the compound to antigen binding to the solid support.

## DISCUSSION

The definition of antigens that are expressed on the surface of cells should provide a basis for a better understanding not only of the host recognition of the fungus but also of the cell wall structure and its role in other cell functions. The properties of MAbs make them extremely useful tools not only for examining the immunodeterminants that are present on the surface but also for investigating the regulation of antigen expression. In this study, an antibody directed against a soluble constituent of the cell wall was used to locate, at least partially, the determinant in the structure of the wall and to describe its expression by cells in different growth states, conditions, and morphologies.

Several observations suggested, although did not prove, that the antigenic determinant was carbohydrate rather than protein (Fig. 3 and 4). The presence of this determinant on the surface of cells of *C. albicans* was indicated by a positive indirect immunofluorescence assay. The antigen was expressed on all isolates of *C. albicans*, *C. stellatoidea*, and *C. guilliermondii* that were tested, although a variable frequency of positive cells was observed. Of the six isolates tested, a variable frequency of cells that strongly expressed antigen was observed among the four positive isolates of *C. tropicalis*. No isolates of *C. parapsilosis*, *C. krusei*, *T. glabrata*, or diploid and haploid strains of *S. cerevisiae* strongly expressed the antigen at the surface. The pattern of strong reactivity of MAb24 among those species examined was similar to that observed in the same species for antigenic structure 4 with absorbed polyclonal sera (34).

The expression of the antigenic determinant was dependent on the growth conditions, growth state, and morphology of the cell (Table 1). The majority (92%) of stationary-phase cells grown at 28°C expressed antigen independent of the two growth media employed. When the temperature at which the cells were grown in the amino acid-supplemented medium was increased to 37°C, however, the proportion of stationary-phase cells that expressed antigen was reduced by almost half, from 92 to 39%. However, the percentage of exponentially growing cells that expressed antigen was similar at both growth temperatures (45 to 49%). There appeared to be no correlation between the budded state of the cell and antigen expression. Budded cells were observed in which only the parent or bud expressed antigen. Thus, about half of the growing cells expressed antigen, while the expression of antigen on nongrowing, stationary-phase cells was dependent on the growth temperature. The difference in antigen expression between growing and stationary-phase cells observed at 28°C means that as cells reach the end of the exponential phase of growth and enter the stationary phase, the number of cells that express antigen increases. Results of preliminary experiments suggest that this increase occurs during the last one or two generations rather than after the cessation of cell division. Antigen expression on germ tubes was restricted to the parent yeast portion and did not appear on the forming germ tube or hypha. Since the mannose composition of cell walls remains fairly constant in growing and stationary-phase yeast cells and in germ tubes (28), only a particular antigenic structure and not the overall polysaccharide would be subject to variable expression.

Since stationary-phase yeast cells grown at 28°C were

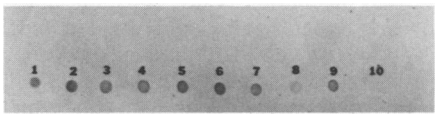


FIG. 4. Immunoblot analysis of immunoreactivity of treated antigen preparations. Portions of antigen were treated as described in the text, and 0.4 μg of treated antigen was spotted onto nitrocellulose paper and was reacted with MAb24. Antigen was treated as follows: spot 1, untreated control; spots 2 to 4, incubated in buffer without reagents at pH 7, 8, and 4.8, respectively; spot 5, incubated with trypsin; spot 6, incubated with α-chymotrypsin; spot 7, incubated with trypsin–α-chymotrypsin; spot 8, incubated with nonspecific protease; spot 9, incubated with α-mannosidase; spot 10, incubated with periodate.

used for the induction of morphogenesis, the high level of antigen expression by the parent portion was consistent with the retention of antigen by the parent cell. The retention of antigen expression by inoculum cells was confirmed by following the expression of antigen on the parent and germ tube portions during morphogenesis (Fig. 2). When stationary-phase yeast cells grown at 28°C resumed growth by budding, the expression of antigen by cells in the resultant exponential-phase culture was reduced. This reduction could be accomplished initially by one of either two mechanisms: (i) dilution by antigen-negative daughter cells or (ii) loss by the inoculum cells themselves. In the first case, the reduction in antigen expression should require one or more generations, depending on the frequency of expression by daughter cells. Examination of the decrease in the proportion of cells that expressed antigen suggested that the second possibility was the most likely one. We have previously shown (6) that when stationary-phase cells are suspended at 37 or 28°C to resume growth by budding, the first budded cells do not appear until approximately 90 or 120 min and maximum budding is not reached until 150 and 300 min, respectively. We have reported (7) that under these conditions in an exponential-phase culture, about 100 min is required to complete bud formation. Consequently, a reduction in cells that express antigen as a result of dilution would not be expected to begin before 190 to 220 min following inoculation. However, the reduction in antigen-expressing cells began at approximately 120 min and reached the levels associated with growing cells well before one cell division. These observations suggest that antigen is shed from the cell, eclipsed, or internalized. The difference in antigen expression of cells that resume growth by bud or germ tube formation is further emphasized by comparison of antigen expression on the small proportion of cells that do not form germ tubes in germ tube-induced cultures. Those cells which formed germ tubes retained antigen expression, while those cells which did not showed a decrease in the number of cells that expressed antigen. This decrease could be accomplished by one of either two mechanisms. (i) There could be a common mechanism, as discussed above, that is shared by all yeast cells that resume growth. (ii) An alternative would be that germ tubes form preferentially from antigen-expressing cells, with a concomitant enrichment of antigen-negative yeast cells. In the latter case, both mechanisms might be operative since yeast cells that express antigen continue to decrease in number after germ tube formation is complete (6).

The expression of the immunodeterminant of MAb24 was variable and sensitive to the growth conditions, growth state, and morphology of the cell. Since the antigenic determinant is most likely part of the carbohydrate component of the mannoprotein, these observations suggest that the structure of the polysaccharide is not constant and is subject to regulation. Such regulation could involve mechanisms of synthesis and loss by degradation or cell wall reorganization. The latter case could result in the masking or eclipsing of the antigen or the shedding of the antigen to the environment.

In addition to the variable expression of the MAb24 determinant reported here, it has been shown (2, 14) that the antigenic determinants of two other MAbs have variable expression, as analyzed by indirect immunofluorescence. Brawner and Cutler (2) have reported an antigenic determinant that is expressed on 50% of the growing yeast cells but on fewer nongrowing cells. This H9 antibody was also able to agglutinate cells from isolates of *C. stellatoidea*, *C. tropicalis*, and *T. glabrata*, but not from isolates of *C. parapsilosis*, *C. krusei*, or several other fungi, including *S. cerevisiae*. An additional C6 antibody showed a similar species reactivity with all but *T. glabrata* (3). Transmission electron microscopy showed that both antigenic determinants, when expressed, were located in the outer flocculent layer (3, 4). The polysaccharide determinant of H9 was lost from the parent cell during germ tube formation and was expressed on the hyphal tip, while the polysaccharide determinant of C6 was expressed on both the parent cell and germ tube (3, 4). Hopwood et al. (14) have reported that the expression of a polysaccharide determinant found on at least two different molecules was expressed generally on approximately 90% of yeast cells, with the parent cells in growing populations expressing the antigen more frequently than did the buds. Indirect immunofluorescence showed that on germ tubes the antigen was expressed on the parent cell and the distal tip. In addition, yeast cells of isolates of *C. tropicalis*, *T. glabrata*, and *C. guilliermondii* also expressed the antigen. The two MAbs reported by Miyakawa et al. (23) reacted by agglutination or immunofluorescence assays with *C. albicans* serotype A, *C. tropicalis*, and *T. glabrata* or with *C. albicans* serotypes A and B and *C. tropicalis*. While the patterns of epitope expression make it unlikely that the antigenic determinant of MAb24 is the same as any of these, Miyakawa et al. (23) have suggested that one of their antibodies and the H9 antibody reported by Brawner and Cutler (2) might have the same determinant. Together, in these studies (2–4, 14, 23) and in the present study, several different antigenic determinants have been examined. However, when similar parameters are examined, there are several observations that are common from the results of the various studies. (i) Antigen expression is dependent on growth state, growth conditions, and morphology of the cell. (ii) There is a heterogeneity of expression among cells in a population. (iii) Thus far, when tested, the epitope is shared by various isolates of *C. albicans*, *C. stellatoidea*, *C. tropicalis*, and *C. guilliermondii*, but not by isolates of *C. parapsilosis*, *C. krusei*, or *S. cerevisiae*. (iv) The expression of antigen may vary among antigen-positive isolates of the same species.

As yet, the observations are not sufficient to determine the extent of variation in the antigenic surface of *C. albicans*. Since in those studies in which they were examined the antigenic determinants were thought to be carbohydrate structures, the variability of protein epitopes at the surface is unclear. We are presently obtaining antibodies from group 3 hybridomas in our collection which are suspected of being directed against a protein determinant. However, these observations do pose the possibility of regulation and dynamics in the surface structure and organization of the cell wall. Such changes may be important in both host-parasite interactions and in the regulation of cell wall structure and form.

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