Variability in surface antigen expression of candida albicans
by Diane Lynn Brawner

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University

Abstract:
Two agglutinating IgM monoclonal antibodies have been produced to two distinct surface antigens on Candida albicans. The expression of these antigens is variable during growth of the yeast and mycelial forms of the fungus. In general, cells in early-logarithmic phase are less reactive with monoclonal antibody than cells in mid-logarithmic phase in yeast form cells. Germinating cells express antigens specific for one monoclonal antibody on mother cells and germ tubes during growth although the quantity of antigens varies with growth phase. The second antigen is expressed on mother cells in early germination but disappears from the mother cell during growth and is expressed only on germ tubes. The antigen reappears on mother cells after 20 h growth and is simultaneously expressed on both mother cell and hyphae. The presence of antigens was established using agglutinination techniques. The location and distribution of antigen was determined using transmission and scanning electron microscopy and immunolabeling with colloidal gold-conjugated and ferritin-conjugated antibodies. The value of these monoclonal antibodies as a serological diagnostic tool for candidiasis was investigated. The antigens which are specific for the antibodies were purified by protease digestion and chromatography and chemically characterized as mannan complexes by UV spectroscopy, gas-liquid chromatography, and mass spectroscopy.

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Diane Lynn Brawner

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ABSTRACT

Two agglutinating IgM monoclonal antibodies have been produced to two distinct surface antigens on Candida albicans. The expression of these antigens is variable during growth of the yeast and mycelial forms of the fungus. In general, cells in early-logarithmic phase are less reactive with monoclonal antibody than cells in mid-logarithmic phase in yeast form cells. Germinating cells express antigens specific for one monoclonal antibody on mother cells and germ tubes during growth although the quantity of antigens varies with growth phase. The second antigen is expressed on mother cells in early germination but disappears from the mother cell during growth and is expressed only on germ tubes. The antigen reappears on mother cells after 20 h growth and is simultaneously expressed on both mother cell and hyphae. The presence of antigens was established using agglutinination techniques. The location and distribution of antigen was determined using transmission and scanning electron microscopy and immunolabeling with colloidal gold-conjugated and ferritin-conjugated antibodies. The value of these monoclonal antibodies as a serological diagnostic tool for candidiasis was investigated. The antigens which are specific for the antibodies were purified by protease digestion and chromatography and chemically characterized as mannan complexes by UV spectroscopy, gas-liquid chromatography, and mass spectroscopy.

INTRODUCTION

Candida albicans is a dimorphic fungus capable of growth as yeast (budding, ovoid cells), pseudohyphae (chains of budding yeast), or, more rarely, as mycelium (tubular, branching filaments) depending on environmental conditions such as temperature and nutrition. Although the organism is generally an unremarkable member of the human microbial flora (107, 134), as an opportunistic pathogen it can promote infections ranging from mild, superficial cutaneous irritations to life-threatening systemic disease (107). In fact, *C. albicans* is currently the most common causative agent of opportunistic fungal infections and has emerged as a very important etiological agent of nosocomial infections in immunosuppressed people (9, 86). Increased incidence of candidiasis is also associated with augmented usage of antimicrobial chemotherapy and prosthetic devices (1, 38, 61, 97, 107, 134).

Other dimorphic pathogenic fungi like *Histoplasma capsulatum* and *Blastomyces dermatitidis* occur in nature and at 25°C in the laboratory as mycelium (M form), are rarely induced to yeast (Y form) *in vitro* (by nutrient and temperature shift), and are found in human lesions predominantly as yeasts (61, 134). One may not be able to

draw direct parallels between other dimorphic fungi and C. albicans because C. albicans exists as a yeast at 25°C and germinates (M form) at 37°C. An argument flourishes over the basic question of whether the Y or the M form of C. albicans is responsible for candidiasis and attests to the impressive lack of knowledge concerning the organism. Historically investigators associated parasitism with the M form and the saprobic state with the Y form (107). However, others contest that this is an oversimplified view for several reasons. First, Y and M forms are usually found in tissue lesions (107); second, several reports indicate that only Y form cells are required for pathogenesis (129, 175); and third, other yeast (e.g. Torulopsis glabrata and Cryptococcus neoformans) are human pathogens but are incapable of M formation (134). Theoretically, mycelial formation does endow the organism with certain advantages which may enhance pathogenicity: 1) the M form is resistent to phagocytosis (28, 32, 33, 81), and 2) hyphae confer a type of mobility which could enhance invasiveness and tissue penetration (107).

The immune competent host, probably through a multitude of mechanisms, is capable of resisting candidiasis. Many investigators, with limited success, have attempted to define the various innate and acquired host mechanisms involved in resistance. Evidence suggests

that macrophages and PMNs ingest and kill C. albicans in vitro. Granulocytopenia and macrophage deficiencies increase susceptibility to candidiasis (28, 77, 81, 117). Chitan and chitosan, when injected intraperitoneally into mice, are immunopotentiating and protect against subsequent lethal challenge with C. albicans by increasing the number of "activated" polymorphonuclear cells in circulation (154). Recent reports also suggest that complement factors play an important role in innate resistance (55, 100, 116).

A major thrust in current research of host defense in candidiasis is directed at the function of the cell-mediated arm of immunity (CMI) and much evidence strongly implies that these factors are of primary importance (16, 27, 67, 68, 69, 114, 139, 164, 174). Extracts of C. albicans cell wall contain antigens which are capable of stimulating a specific immune response (37). Purified polysaccharide antigens have been reported to induce strong mitogenic responses in vitro, causing the proliferation of T cells. The stimulated T cells produce a T cell replacing factor which has been reported to stimulate B cells in vitro (119, 120), and antibody response is dependent upon this T-B cell interaction (121). Patients with T cell deficiencies, e.g. thymoma, or DiGeorge and Nezeloff syndromes, are more prone to

candidiasis (107). Reconstitution with T cells or transfer factor can ameliorate symptoms in some of these patients (70, 113). In chronic mucocutaneous candidiasis (CMC), mannan in serum has been shown to specifically inhibit C. albicans-induced lymphocyte proliferation and has been correlated with activation of T suppressor cells (40, 41). Durandy (37) has suggested that mannans may act as haptens because anti-mannan antibody can only be elicited if mannan is coupled with methylated bovine serum albumin. Although these data strongly implicate CMI as the major line of host defense in CMC, other defense mechanisms are required against non-CMC candidiasis (107). First, patients with CMC do not succumb to deep-seated, disseminated Candida infections. Second, not all CMC patients have T cell deficiencies and third, nonspecific T cell stimulation does not always correlate with increased resistance.

Except for early poorly defined studies, few investigators have examined the role of antibody in host defense against candidiasis. The general dogma contends that C. albicans-specific antibody is not protective because most candidiasis patients are not only B cell competent but often develop high precipitin and agglutinin titers against the fungus (45, 68, 77, 106). In addition, levels of antibody to Candida are often comparable in

healthy and infected individuals (62, 71, 96). However, several investigators (35, 101, 103, 115, 116) have presented evidence that humoral immunity may contribute to protection, and resistance may be passively transferred by serum factors from immune to nonimmune mice (115). Others have recently presented evidence to indicate that T-B cell interactions are required for immunity to candidiasis (37, 174). B cell deficient mice are more susceptible to candidiasis (29, 107). Furthermore, since investigators generally agree that neutrophils are important in defense against candidiasis (107), the finding that serum depleted of specific antibody by absorption with C. albicans cells promotes phagocytosis less than nonabsorbed serum (116) provides indirect evidence that antibody is important in resistance. Confusion concerning the role of antibody and the vague understanding of host-parasite relationships in candidiasis may be the result of the lack of understanding of the characteristics and composition of the cell surface of Candida. Using polyclonal antisera to serologically classify yeasts, investigators reported that antigen may not be uniformly expressed in all strains of C. albicans (5, 50, 51, 98, 101, 115, 124, 125, 157, 163). Additionally, individual strains of yeast express antigen variably as a function of their metabolic state or morphologic form (60, 87, 88, 135, 145, 153, 161), but the

location of these variable determinants has not been investigated. From the standpoint of host-parasite encounters, the definition of surface changes seems imperative. Surface antigens make initial contact with host immune mechanisms during candidiasis. However, antigens may not be equally expressed among strains of Candida or may vary from strain to strain (124, 161). Results of recent studies indicate that a single strain of C. albicans may undergo antigenic variation during pathogenesis of candidiasis (124).

Studies have been done to define the process of dimorphism. A few attempts have been made to correlate morphogenesis in C. albicans with metabolic pathways involving glucose (75), levels of cyclic nucleotides (112), and changes in cytoplasmic proteins produced concomitant to conversion from Y to M forms (13, 15, 87). The latter studies were modeled after the work of others who had demonstrated changes which occur in protein synthesis in Y to M transitions in the dimorphic fungus Mucor (60, 159).

Numerous additional studies have been done to define the factors which initiate and control morphogenesis in C. albicans, and the literature is replete with conflicting data and conclusions for several reasons. First, not all strains of Candida behave similarly under the same

conditions. Second, C. albicans is capable of existing in forms intermediate between yeast and hyphae, all of which may occur simultaneously; namely, as budding yeast, chains of yeast, chains of elongated yeast, germ tubes without constricted bases, germ tubes with constricted bases or yeast cells with multiple germ tubes, one of which may be constricted and the other not. Terminology used to describe these structures include hyphae, pseudohyphae, germ tubes, filaments, and pseudogerm tubes, and these terms are often used interchangeably by some investigators resulting in added confusion and difficulty in comparing and interpretting reports. Third, variability in growth conditions used to control and induce Y to M conversion has also led to confusion. Consequently, many substances which have been reported to prevent (or promote) M conversion are subsequently refuted by other investigators studying the same parameter under dissimilar conditions. For example, one group reported that high glucose concentrations in a yeast-inducing medium enhanced germination (75) while others report no enhancement by glucose (39, 105). Factors reported to affect morphogenesis include cell size and temperature shifts (14, 76, 89, 165), differences in cell wall and cell membrane composition (172), pH of growth environment (105), availability of sulfhydryl groups (105),

concentration of metal ions such as zinc and cobalt (53, 165, 177), cyclic nucleotides (112), proline (91), glucose (75), biotin (176), amino acid concentration (142, 190), and released metabolites (e.g. tryptophol and phenylethyl alcohol (53, 80, 142). Unfortunately, in many cases, for each investigation which concluded that one of the above products or environments enhanced or inhibited germination, a subsequent study done using different experimental parameters would refute the previous findings. Researchers, examining factors responsible for morphogenesis, have employed a multiplicity of environmental and physiological variables to induce growth of the altered phenotype, and induction promoted by many of these variables may generate fundamental changes in cellular metabolism or enzyme functions which incidentally parallel, but are uninvolved in dimorphism.

An interesting alternative for controlling germination may be through the use of germination inhibitory factors which are produced by fungi including C. albicans (39, 54, 79, 98). These regulators have been largely underrated for their potential impact on defining morphogenesis. Recent work (52, 53, 54) has led to the isolation, purification, and partial characterization of an autoregulatory factor termed morphogenic autoregulatory substance (MARS), a low molecular weight (10,000 d),

heat-stable substance produced and released by C. albicans in vitro. MARS can be purified from cell filtrates of C. albicans and C. tropicalis but not from other Candida sp.. Preliminary identification indicates that the substance is a heterocyclic compound, and perhaps a proline analog or derivative. The advantage of using this compound to control germination is that no other artificial nutritional or temperature shifts are introduced into experiments to suppress germination.

Investigations done to compare other aspects of Y and M metabolism and morphology have been less ambiguous. Protein synthesis occurs at similar rates in both Y and M forms (31) and cytoplasmic protein synthesis, but not mitochondrial protein synthesis, is required for phase shift (142). To examine the possibility that proteins are expressed uniquely by each form, one and two-dimensional (2-D) gel electrophoreses have been done on proteins from Y and M phases. Dabrowa (31) reported 2 protein bands unique to Y forms and a single band unique to M forms, while in other studies, mycelial cells were found to have fewer proteins than yeast cells but no proteins unique to M forms could be detected by 2 D gel electrophoresis (13, 15, 87, 88, 89). Although no major protein differences can directly account for morphogenesis, investigators have not ruled out the possibility that conversion from Y to M

may be concurrent with post-translational modification of proteins (88) or differential gene expression (13).

Notable differences exist in the content of phospholipid and sterol concentrations of Y and M forms of C. albicans. Lipid synthesis is lower in M then in Y cells (152). However, yeast cell membranes contain phosphatidyl serine, phosphatidyl inositol, sphingolipids, phosphatidyl ethanolamine, and phosphatidyl choline while only phosphatidyl ethanolamine and phosphatidyl choline have been found in exponential M phase cells (3, 91).

In contrast to biochemical studies of C. albicans, little has been done to establish the nature or organization of the yeast genome. C. albicans has no known sexual cycle (94) yet strong evidence has been presented recently which indicates that many, if not all, clinical isolates are diploid (170, 171). While others have suggested that C. albicans strains are haploid (126, 136), support for the diploidy concept has been strengthened by DNA content data (119, 111, 170) and DNA reassociation experiments (132). Although Olaiya (111) reports that germination may be a sexual characteristic of C. albicans, the relationship has not been universally accepted (126, 136) and no other definite relationships between nucleic acid synthesis and morphogenesis have been confirmed. Because a sexual stage of C. albicans has not

been found, investigators have used artificial spheroplast fusions (126, 136) and auxotrophic mutants (64, 73, 127, 169) to study genetic mechanisms involved in pathogenesis and dimorphism.

Despite numerous studies, little is known about the mechanisms by which germination occurs in C. albicans. Investigators (4, 58) have reported that the initial cell size and volume of the C. albicans mother cell prior to either bud or germ tube formation is the same and growth rates of the 2 cell types are comparable. Yet the cell volume of the bud is 27% greater than germ tube volume throughout growth. Unlike other filamentous fungi, where protoplasmic volume and hyphal length both increase exponentially during growth (160), the initial increase in length of developing mycelia in C. albicans is linear and cytoplasmic volume remains constant during hyphal development (46). Recently a mechanism was proposed to explain the physiological changes that occur when the Candida cell undergoes germination. Gow and Gooday (47) proposed that early onset vacuolation occurred in mother cells because the growth-supporting cytoplasm, which remains at a constant volume during the development of young germlings (linear growth kinetics), is extruded into the germ tube. Regeneration of cytoplasm occurs during a

delay in cell growth that occurs prior to septation, branching, and secondary germ tube formation.

Comparisons of the composition of Y and M cell walls have been informative. Yeast cell walls, which function to maintain the shape of yeast cells and serve as the point of contact with the environment, undergo metabolic turnover during growth and cell division (107). These changes in the cell surface remain undefined but stationary cells are more resistant to enzymatic hydrolysis than actively metabolizing cells which indicates that cell wall polysaccharides become extensively linked with age (13, 107). C. albicans cell walls are chiefly composed of glucan, mannan, protein, and small amounts of lipids (4, 23, 66). The chemical analysis of yeast and hyphal cell walls has demonstrated only quantitative but not qualitative differences (10, 23, 66). Mannan components of the cell wall have received the most attention and consist of an (alpha 1-6) linked mannopyranose backbone modified by side chains of 6 or fewer (alpha 1-2)-linked hexose subunits (8, 63, 118). Differences between mannans is thought to provide the basis for serotyping of C. albicans A and B (72, 118, 149). Many cell wall mannans are modified by proteins which are linked via N-acetylglucosamine residues. Cell wall glucans are composed of (beta 1-6)-linked glucopyranose with (beta 1-3) branch points.

Several investigators have examined the ultrastructure of the cell wall of C. albicans and report that it is composed of from 5 to 8 layers (34, 123, 137). Cassone (21) has located mannan throughout all layers of the yeast cell wall. Poulain maintains that mannan and glucan are confined to layers 1-4 and that the majority of cell wall chitin exists in layers 5 and 7. With aging, layers 1 and 3 disappear (123, 161). Electron micrographs of the cell wall show that the outermost granular layer thins out over the germ tube. A second layer and third inner layer, of high and medium electron density, respectively, cover both the mother cell and germ tube in the same density and thickness. A fourth layer of low electron density is juxtaposed to the plasmalemma. The thickness of this layer is greatly increased on germ tubes as compared to mother cells. Concentrations of mannan and glucan in Y and M forms are comparable but germinating cells contain only a third as much glucosamine (presumably in the form of chitin) as yeast cells (23). The latter is supported by observations (20) of an increase in width of an electron-transparent layer in germ tubes. This layer correlates with the third layer reported by Schwertiz (137), and is composed of chitin. An increased uptake of N-acetylglucosamine concomitant with increased chitin synthesis and its deposition at the apices of germ

tubes in the mycelial form and at the bud site in yeast form, has been reported (10).

Rates of chitin synthesis vary between Y and M cells and the key enzyme involved in the production of chitin from glucose, L-glutamine-D-fructose-6-phosphate aminotransferase, has both a higher specific activity in germinating cells and its induction is correlated with the appearance of germ tubes (26). Chitin synthetase, the terminal enzyme in the pathway for chitin synthesis, has a higher specific activity in M cells and, in fact, M cells incorporate 10 times the amount of GlcNAc; and, although the data is disputed by some investigators (166), this precursor has been demonstrated to induce germ tube formation (92, 142, 143). Recent evidence indicates that hyphal formation *in vitro* may occur simulaneously with the export of hydrolytic enzymes (e.g. N-acetylglucoseaminidase into the surrounding medium (150). The enhanced export of these enzymes is inducible with GlcNAc and may be a virulence factor (92).

Cell surface glycoproteins have been implicated in adherence of yeast cells to mammalian host cells (78, 85, 161. When chitan or its derivative chitosan are removed or blocked on the surface of *Candida*, *in vitro* adherence is inhibited (78). Adherence to acrylic surfaces is substantially enhanced by the presence of a preformed outer layer which is synthesized in response to

appropriate carbohydrate concentrations in growth medium and is growth-phase dependent (93). Adhering cells develop a fibrillar layer which is composed of polysaccharide granules that are also reported to be involved in adherence (161).

C. albicans strains have been grouped according to antigenic types by several workers (50, 51, 162, 163). Hasenclever divided the yeast in 2 serogroups, A and B. Group A possesses the same complement of surface antigens found on group B plus 2 additional antigens. Hasenclever (50, 51) and others (149, 156) reported close antigenic relatedness between strains of C. albicans Group A and C. tropicalis and between C. albicans group B and C. stellatoidea [referred to by some as C. albicans var. stellatoidea based on this close relationship and the fact that this organism also produces germ tubes and differs biochemically from C. albicans only in its inability to ferment sucrose, (94)]. A third serogroup of C. albicans has been reported (104) but is not universally accepted (107). Differences in mannan structure, the extent of branching, length of side chains and particularly the proportion and position of (alpha 1-3) bonds in side chains has been reported to be, at least in part, responsible for the antigenic differences between serogroups A and B (107). Strains of C. albicans have

been ranked in 3 groups according to their relative virulence (101). However, Hasenclever (50) could not distinquish virulence differences between serotypes A and B which indicates that the effects of strain variations on virulence are minor in comparison with species variation.

With the exception of A and B serotyping of C. albicans rapid tests to differentiate strains of the species are not available. Several methods which are costly and laborious have been described. An enzymatic profile analysis which involves testing for 19 enzyme activities has been described (19). The resistogram method of Warnock (168) and Meinhof (95) is a method of strain differentiation based on resistance of C. albicans to selected biochemicals. Another method of differentiation depends upon killer strains of S. cerevisiae which produce an undefined substance that is lethal for selected strains of C. albicans (122). Particularly for epidemiological and diagnostic purposes a rapid system for grouping and identifying specific strains of C. albicans would be helpful.

The development of rapid diagnostic tests for distinquishing superficial from disseminated forms of candidiasis also have been unsuccessful. Oral, vaginal and cutaneous candidiasis present few problems in diagnosis, because lesions are usually obvious and the fungus is easily cultured. However, patients with

disseminated candidiasis do not present with pathognomonic symptoms and blood cultures are often negative. Therefore, attempts have been made to diagnose disseminated disease by seroassay (for antibody specific for C. albicans). Unfortunately, the presence of C. albicans on mucosal surfaces of colonized (non-diseased) individuals promotes antibody production (7, 65). Therefore, unless semi-quantitative determinations or serial titers of antibody are done, serodiagnosis is a meaningless indicator of infection. These techniques are also crude in that, presently, tests often rely on poorly defined and unstandardized antigen preparations which preclude reliability. Finally, tests to detect serum antibody may be falsely negative in immunosuppressed people, the population which presently is in greatest danger of disseminated candidiasis.

Detection of candidal antigens and metabolites in serum, rather than antibody, has become popular in diagnosing disseminated disease. Candidal mannanemia (44, 131, 138) and increased arabinitol levels (131) have been found in sera of patients infected with C. albicans. However, renal insufficiency which occurs in many diseases besides candidiasis has been shown to cause elevated levels of arabinitol (131); therefore care must be taken to distinquish whether accumulations of arabinitol and mannose are due to renal insufficiency from other causes.

One possible variation would involve the use of cytoplasmic candidal antigens which are reported to cross-react less with antibody found in colonized individuals than do surface antigens (65). However, cytoplasmic antigen preparations are often contaminated with surface antigens (6). A possible limitation of the antigen detection technique is that circulating immune complexes of C. albicans antigen and antibody have been sporadically reported and may inhibit antigen detection (17, 130).

In my work, monoclonal antibodies have been developed which are specific for cell surface determinants of C. albicans. These antibodies have been used to define and characterize the antigenic mosaic surface of the fungus in both yeast and mycelial forms. I have also used 2 monoclonal antibodies, in conjunction with transmission immunoelectron microscopy and background electron imaging techniques (BEI), to locate and semi-quantify the specific surface determinants as well as to study their dynamic expression during Y form growth and during conversion from Y to M form. In addition, these antibodies were used to develop an immunodiagnostic test to detect the presence of candidal antigen in human and animal sera and to compare taxonomic markers among various strains and genera of yeast.

The antigenic determinants which specifically reacted with two monoclonal antibodies were extracted from the

cell surface of C. albicans, purified and biochemically characterized by gas liquid chromatography and mass spectroscopy.

MATERIALS AND METHODS

Organisms and culture conditions.

Hybridoma production, agglutination tests, growth curve analyses, media comparison studies, immunofluorescent antibody labeling, and yeast phase antigenic variability studies. Candida albicans 9938 (Mycology Unit, Tulane Medical School, New, Orleans, LA) was used to immunize mice. Other organisms used in either agglutination or growth curve studies were: C. albicans 394 (R.P. Morrison, University of Oklahoma, Norman, OK); and 72 clinical isolates of C. albicans which were gifts that have subsequently been incorporated into the Montana State University mycology stock collection. A single strain of C. albicans var. stellatoidea, 7 strains of C. tropicalis, 3 strains of Torulopsis glabrata, and single strains of C. parapsilosis, C. krusei, Cryptococcus terreus, C. neoformans, Saccharomyces cerevisiae, Rhodotorula sp., Trichosporon beigelii, Phaeococcmyces exophialae and Blastoschizomyces pseudotrichosporon were from the Montana State University mycologic collection. Autoagglutination, particularly prevalent in strains of C. tropicalis masked agglutinin reactivity and prevented the use of 39 additional strains of yeast. The identification of each organism was reconfirmed using API 20C Yeast

Identification Strips (Analytab Products, Plainview, NY) or conventional nitrate assimilation and carbohydrate fermentation tests. Germ tube tests were positive on all strains of C. albicans. The fungi were grown at 22-24°C on potato flakes agar slants (PD, 133). Cells were harvested from the slants, washed twice in sterile distilled water and maintained in suspensions of sterile distilled water at 4°C.

Organisms were grown in either glucose (2%, Baker, Phillipsburg, NJ), yeast extract (0.3%, BBL, Cockeyville, MD), peptone (1.0%, Difco, Detroit, MI) broth (GYEP) or brain heart infusion broth (BHI, Difco, Detroit, MI). Additional media utilized in comparison studies included potato dextrose (PD) broth and agar, Sabouraud dextrose (SAB) broth and agar, brain heart infusion agar (Difco), synthetic amino acid medium broth, the same medium solidified with agarose (SAAM, GIBCO Diagnostics, Madison, WI) and a chemically defined medium, synthetic amino acid medium-fungal broth (SAAMF, GIBCO) and SAAMF solidified with agarose (SAAMF, GIBCO). All broth cultures were rotated during growth (New Brunswick Shaker Incubator, 160 rpm).

Antigenic variability studies in morphogenesis. C. albicans 105, a recent clinical isolate which has been incorporated into our culture collection, was used throughout the study and was chosen for its ability to

germinate at levels of >90% in a defined germination medium GM-2 (54). Germ tube tests were consistently positive (92-98% by 3.5 h) in both human serum and GM-2. The fungus was stored at 4°C in distilled water subsequent to growth on potato flakes agar (133). For immunoelectron microscopy studies the organisms were grown to stationary phase in glucose (2%, J.T. Baker Chemical Co., Phillipsburg, NJ)-yeast extract (0.3%; BBL Microbiology Systems, Cockeyville, MD)-peptone broth (1.0%; Difco Laboratories, Detroit, MI). After washing in 0.01 M phosphate buffered saline (PBS) pH 7.2 (warmed to 37°C), cells were inoculated (5×10^5 organisms/ml) into 100 ml of GM-2 (described above) in 125 ml Erlenmeyer flasks (53). Germination was suppressed in control flasks either by the addition of a crude extract of the morphogenic autoregulatory substance (MARS) (54) or by inoculation of 1×10^8 cells/ml to the GM-2-containing flasks. All flasks were incubated at 37°C with rotation (160 rpm).

Serotyping of yeast strains

Serotyping of 6 strains of *C. albicans* was done according to the methods of Hasenclever (50). In brief, a known serotype A (ATCC 28367) and serotype B (ATCC 38696) were grown in GYEP broth, washed, and heat-killed (60°; 30 min, monitored by plating for viability). Known type A

antiserum A207 and type B antiserum 526, obtained from Hasenclever's original C. albicans antisera collection, were each absorbed 4 times as follows: one ml (in duplicate) of each serum was absorbed with 1 ml of packed serotype A or serotype B cells. The first and third absorptions were done at 45°C for 4 h and the second and fourth were done at 0-4°C overnight. The four absorbed antisera were diluted 1:30 in saline and serial two-fold dilutions were made from the 1:30 dilution. The organisms to be serotyped were grown in GYEP broth, washed, heat-killed (60°C for 30 min), counted (hemocytometer) and diluted to 5 x 10^8 cells/ml. Tube agglutination tests were performed with these organisms using the absorbed antisera (43).

Immunization of mice

Whole cell Candida albicans immunizations. A protocol which promotes agglutinin responses in mice (173) was followed to immunize BALB/cByJ mice which were obtained from Jackson Laboratories (Bar Harbor, ME). This entailed 6 weekly intraperitoneal 0.4 ml injections of a saline suspension of heat-killed C. albicans 9938 blastoconidia (10^8/ml). Three days after the last booster injection, mouse sera were checked for C. albicans-specific agglutinins, spleens were removed from agglutinin-positive animals and splenocytes were prepared for cell fusion.

Immunization with candidal ribosomal antigen fractions. Ribosomal fraction antigens (rAg) had been prepared previously (1000 ug/ml, 173) and stored at at -70°C. Equal volumes of rAg and complete Freund adjuvant in volumes sufficient to administer 100 ug antigen per 0.2 ml injection per mouse were placed into separate syringe barrels. The adjuvant-antigen emulsion was thoroughly mixed (double-butted syringe) until the mixture formed a very stiff emulsion which did not disperse when placed onto the surface of water. Mice were injected subcutaneously at the nape of the neck on day 1. Ribosomal-adjuvant suspensions were made on days 16 and 36 (as above) except incomplete Freund adjuvant was used and the emulsion was injected as on day 1. Spleens were removed from mice on day 42 and splenocytes were prepared for fusion to myeloma cells.

Hybridoma production

The hybridoma techniques utilized were those of Chesebro, et. al. (25) with minor modifications. Maintenance and selective media were supplemented with amphotericin B (1 ug/ml), penicillin (100 U/ml) and streptomycin (100 ug/ml, Irvine Scientific, Santa Ana, CA). Cell fusions were effected with a 35% (w/v) solution of polyethylene glycol 1540 (Baker, Phillipsburg, NJ) in RPMI 1640.

Agglutinin reactivity was detected directly by combining hybridoma culture supernatant material with heat-killed C. albicans 9938 blastoconidia. Hybridoma antibody production was determined indirectly by using an enzyme immunosorbent assay (BRL Hybridoma Screening Kit, Bethesda Research Laboratories, Gaithersville, MD). All antibody-producing hybrids were subcloned by limiting dilution a minimum of 3 times. Three of the hybrids, C6, B6, and H9, produced antibody that agglutinated C. albicans 9938. Nine additional nonagglutinating IgM (5), IgG_1 (1), and IgG_2 (3) monoclonal antibodies were also produced, but were not characterized beyond class designation or used in experiments.

Characterization of monoclonal antibodies

Antibody class. Heavy chain specific anti-mouse immunoglobulins (Meloy Laboratories, Springfield, VA) were used in agar double-diffusion (Ouchterlony) and immuno-electrophoretic analysis (43) to identify the antibody class of the agglutinin.

Temperature stability. Monoclonal antibodies C6, B6, and H9 in cell culture supernatant fluid were heated to 60°C for 5 to 30 min, cooled, and reacted with one drop of a suspension of C. albicans 9938 (5×10^8/ml). Agglutinin reactivity was compared to that of unheated monoclonal antibodies. Agglutinins C6, B6, and H9 were

subjected to lyophilization, refrigeration (0-4°C for 1 week), and freezing (-70°C for 1 week). Reactivity was assessed by slide agglutination as described above and compared with nonperturbed hybridoma cell supernatant fluid.

Precipitin tests. Ouchterlony double diffusion tests were done using diluted mouse ascitic fluid containing either of the monoclonal antibodies or 5x concentrated hybridoma cell supernatant fluid (Amicon ultrafiltation membranes, Amicon Corp., Danvers, Ma.) and crude antigen extract which was obtained by methods previously described (11).

Purification of monoclonal antibody

Affinity chromatography. Sepharose 6-B (Pharmacia, Uppsala, Sweden) was washed in distilled water twice (13xg for 5 min). Packed beads (25 ml) were resuspended in 0.5 M carbonate buffer pH 11 and 2.5 ml divinyl sulfone was added. Beads were incubated 70 min at 22-24°C, and washed in distilled water (5xg). Activated beads (20 ml) were added to 10 ml of crude antigen suspension (20 mg antigen in 10 ml carbonate buffer pH 10), incubated overnight at 22-24°C, washed (distilled water), resuspended in 25 ml 0.5 M bicarbonate buffer pH 8.5 in 2% beta mercaptoethanol (2 h at 22-24°C), washed, and packed into a glass column (5.0 x 1.5 cm). The columns were equilibrated with 0.1 M

PBS pH 7.4 at 22-24 ^{o}C. Five ml of ammonium sulfate-concentrated antibody was added to the column and the column was washed in 1 bed volume of PBS. As a control, activated beads which had not been treated with crude antigen extract were reacted with monoclonal antibody solutions. Eluant fluids were collected and tube agglutination tests were done on the eluates.

Ascites production

Production of normal mouse ascitic fluid. Mice were injected intraperitoneally (IP) with 0.5 ml of 2,6,10,14 tetramethylpentadecane (Pristane, Sigma Chemical Corp., St. Louis, MO) 10 days prior to injection with 2.5 x 10^6 S180 or myeloma cells in 0.2 ml RPMI 1640 (Gibco Laboratories, Grand Island, NY). Approximately 75% of the mice produced ascites within 6 weeks and the fluid was removed, allowed to coagulate overnight at 0-4^{o}C, centrifuged at 12,000xg for 20 min, and tube agglutination tests using C. albicans 9938 were done to determine agglutinin reactivity against C. albicans 9938. All normal ascitic fluid was absorbed with 10^8 C. albicans whole cells and stored at -20^{o}C.

Production of ascitic fluid which contained monoclonal antibodies. Hybrid (monoclonal) cell lines H9 and C6 were grown in RPMI (Gibco) with 10% FBS for 6 days, washed in fresh RPMI with FBS, counted (hemocytometer) and 2 x 10^6

cells were inoculated (IP) into Pristane-treated BALBc/ByJ mice. Ascitic fluid, which accumulated within 4-6 weeks, was aspirated from anesthetized mice, allowed to clot, and centrifuged at 12,000xg for 20 min. The supernatant fluid was removed, tested for tube agglutination titer against whole cell suspension of C. albicans 9938 (43), and stored at -20°C.

Agglutination tests

Standardization of qualitative and semi-quantitative slide agglutination tests was facilitated by using ring slides (2 cm diameter rings, Scientific Products). Twenty five microliters of the agglutinin in cell culture supernatant fluid, either undiluted, serial two-fold diluted, or ammonium-sulfate precipitated (50% at room temperature prepared by methods similarly used by others, (7) or 25 ul of diluted mouse ascitic fluid (titer = 1280), was added to the ring slide and mixed with one drop of antigen suspension (heat-killed C. albicans 9938 blastoconidia at 5 x 10^8 cells/ml saline) and the slide was gently rocked for 2-5 min. Degree of reactivity (determined by relative degree of cell aggregation, 1-4+) and endpoint titers were determined macroscopically and microscopically. Semi-quantitative tube agglutinin titers were done in serial two-fold dilutions of 0.3 ml total volume with 5 x 10^5 C. albicans 9938 per tube and were

incubated 2 h at 37°C, refrigerated 22 h, and allowed to remain at 22-24°C at least 1 h prior to determining endpoint agglutination titers.

Growth curve analyses

Agglutination studies. To determine if reactivity of agglutinins varied during growth, growth curve analyses were done in duplicate on 13 yeast strains: C. albicans 4, 16, 17, 19, 34, 105, 170, 394, 9938; T. glabrata 15, C. tropicalis 4, C. parapsilosis, and S. cerevisiae. Each strain was first grown in GYEP broth or BHI broth for 48 h. The inoculum obtained from each strain was adjusted at time 0 to give 7×10^6 to 5×10^7 blastoconidia in 200 ml of fresh medium (either GYEP broth or BHI broth) in 500 ml Erlenmeyer flasks. Cultures were incubated at 37°C with rotation (160 rpm). At hourly intervals, broth aliquots sufficient to provide a 5×10^8 cells/ml suspension were aseptically removed, counted (hemacytometer), and reactivity with the agglutinating monoclonal antibody was determined by the slide test.

Morphogenesis studies. Growth curve analyses (as above) were carried out on strains C. albicans 105, 394, and 9938 to determine reactivity of both agglutinins and to establish the optimal initiation point for the study of surface antigen expression with immunolabeling. Agglutinations were done hourly from 3-18 h and at 24, and 48 h,

and the degree of agglutinin reactivity was compared to the amount of colloidal gold bound to the cell surface of the yeast at a corresponding time during growth. Each growth curve experiment was done on at least two separate occasions.

Media comparisons studies

C. albicans 9938, 394, 17, and 34 were chosen to investigate the effects of growth environment on H9 agglutinin reactivity. Each strain was grown in 100 ml GYEP broth and in 100 ml BHI broth in 250 ml Erlenmeyer flasks for 24 h at 37°C while rotating at 160 rpm. The yeast cells were washed in saline 3 times and enumerated (hemacytometer). GYEP broth-grown cells were used to inoculate 10 ml (10^5 yeast cells/ml) of SAB broth in 25 ml Erlenmeyer flasks and agar plates, 10 ml GYEP broth in flasks and agar plates, and 10 ml PD broth in flasks and agar plates. BHI-grown cells were used to inoculate 10 ml of BHI (10^5 cells/ml) and BHI agar plates. Broth flasks were incubated at 37°C and rotated at 160 rpm for 24 h. Agar plates were streaked for the isolation of C. albicans and incubated for 24 h at 37°C. At 24 h, growth was gently swabbed from the surface of the agar plates. After washing 3 times in saline, suspensions

(5×10^8 cells/ml) were made of agar and broth-grown yeast cells for slide agglutination tests using H9 agglutinin.

Soluble crude antigen extraction and absorption

The agglutinin-specific antigens (AgC6, AgB6, and AgH9) were solubilized from whole cells by the following procedure: yeast cells were grown in 500 ml GYEP at 37°C with rotation at 160 rpm for 24 h, washed 3 times in saline, resuspended in 100 ml 1 M NaCl, heated to 68°C for 40 min and centrifuged (1800xg) at 22-24°C. The supernatant fluid was exhaustively dialyzed (as determined by conductivity measurements) against distilled water at 4°C and lyophilized. Antigen preparations (5 mg/ml), resolubilized in 0.15 M NaCl, produced a single precipitin band when reacted with each of the three monoclonal antibodies in an Ouchterlony double diffusion test. All three antigens were contained in the 1M NaCl extract.

Soluble crude antigen extracts were tested to determine their capacity to absorb H9 agglutinin reactivity by combining 0.1 ml of monoclonal antibody solution (agglutinin titer 128) with 0.1 ml antigen extract (5 ml/mg saline) at 22-24°C for 15 min. Tubes were centrifuged at 300xg for 3 min and the supernatant fluid was tested for reactivity by the slide agglutination test.

Several yeast strains which were used in growth curve analyses were also tested for their ability to absorb the agglutinin from solution. Whole cell suspensions were prepared from 24 h cultures (37°C, 160 rpm) in GYEP and in BHI broths. One-half ml of monoclonal antibody H9 was added to the first of three tubes, each of which contained a pellet of 5×10^7 washed yeast cells. The cells in the first tube were resuspended, incubated for 5 min at 22-24°C, centrifuged at 300xg for 3 min and the supernatant fluid was removed and added to the second tube. This procedure was followed through the third tube and the resulting supernatant fluid and the original agglutinin preparation were agglutinin titered against C. albicans 9938 and tested for anti-mu reactivity with goat anti-mouse IgM (Meloy Laboratories, Springfield, VA) in Ouchterlony double diffusion plates.

Indirect fluorescent antibody test

C. albicans 9938 was grown in GYEP broth at 37°C with rotation at 160 rpm. At 5, 24, 48, and 96 h incubation, aliquots of broth culture were removed and the cells were washed in 0.01 M phosphate buffered saline (PBS) pH = 7.2. Twenty microliters containing 2×10^6 C. albicans cells were mixed with 50 ul of monoclonal antibody H9 (agglutinin titer 64) for 30 min at 22-24°C. The cells were washed 4 times in 0.01 M PBS and 20 ul of

fluorescein isothiocynate (FITC)-labeled goat anti-mouse immunoglobulin (Cappel, West Chester, PA) diluted 1:10 in 0.01 M PBS, was added to the cell pellet. After incubating 30 min at 22-24°C, the cells were washed 4 times in 0.01 M PBS and examined using a fluorescent microscope (Leitz Ortholux II, Boyle Instruments, Gig Harbor, WA).

Immunodot-spot assay

Nitrocellulose paper was boiled for 5 min in distilled water, dried, and sectioned into quadrants. One half milliliter of each monoclonal antibody (B6, C6, and H9) diluted 1:10 in 0.15 N saline (final agglutinin titer = 1280) and normal ascitic fluid (diluted 1:10) were spotted onto the nitrocellulose (one spot/quadrant). After drying, nitrocellulose squares were washed in Tris-buffered saline pH 7.4 (TBS: 50 mM Tris-HCl; 200 mM NaCl), and blocked in 3% BSA for 2 h at 22-24°C with rotation (160 rpm in 6 well tissue culture plates (Falcon, Becton-Dickinson, Oxnard, CA). Blocking solution was aspirated from the wells; and (pooled, normal) human test serum or (normal) mouse sera preabsorbed with C. albicans 9938 and spiked with either 5000, 1000, 500, 100, 25, 10, or 1 ng/ml of crude surface antigen extract from C. albicans 9938, was added. A single control well contained only preabsorbed unspiked normal human serum. Plates were incubated with rotation

(160 rpm) overnight at 22-24°C. Human serum was aspirated from the wells and nitrocellulose strips were washed for 30 min with rotation (200 rpm) in 5 changes of TBS. Strips were blocked in 3% BSA for 15 min with rotation (160 rpm). A 1:200 dilution of rabbit anti-C. albicans antiserum (produced as previously described, 50) which had been preabsorbed with mouse spleen cells was made in blocking solution, and 1.0 ml was added to each well and incubated for 2 h at 22-24°C with rotation (160 rpm). Strips were washed 5 times in TBS (as above) and 1.0 ml of a 1:500 dilution in blocking solution of goat anti-rabbit antiserum conjugated with horse radish peroxidase (Cappel, Downingtown, PA) was added to each well for 2 h at 160 rpm and 22-24°C. After washing in TBS, 0.5 ml of substrate [3 mg/ml stock solution of 4-chloro-1-naphthol, (Sigma) in methanol diluted in 5 volumes of TBS] was added to each well and nitrocellulose strips were observed for 2-10 min for the development of insoluble purple pigment at the site of the spotted monoclonal antibodies. In addition to the mock sera, 3 other types of sera were tested for antigen by dot-spot analyses: 1) pooled serum collected pre-mortem from mice injected with lethal doses of C. albicans 9938 (1×10^6 to 4×10^6 blastoconidia), 2) 14 sera from leukemic and oncology patients known to be colonized with C. albicans, and 3) a single serum from a patient known to have succumbed to invasive candidiasis.

The patient's own isolates of C. albicans were obtained at the time of the serum collection. The isolates from all patients reacted 2-4+ with monoclonal antibodies B6, C6 and H9.

Indirect immunoelectron microscopy

Location of surface antigen with ferritin labeling. Yeast cells were grown in GYEP broth for 18 h at 37°C with rotation (160 rpm). Cells were washed in 0.01 M PBS, reacted with 1.0 ml of either H9 monoclonal antibody (agglutinin titer = 12,500) or absorbed (described above) normal ascitic fluid, washed in PBS, and reacted with 0.1 ml of undiluted goat anti-mouse ferritin-conjugated antibody (Cappel) for 30 min at 22-24°C with rotation (160 rpm). Yeast cells were washed in PBS, fixed (0.25% formalin), and embedded in 2% agarose which was subsequently cut into 1 mm cubes. Cubes were post-fixed in 4% OsO_4 for 12 h at 4°C, rinsed in cold water (pH 7.2-7.4), dehydrated through an ethanol series, embedded in Spurr's resin (147), thin sectioned, post-section stained with lead citrate and uranyl acetate, and examined in a Zeiss EM9S-2 electron microscope.

Antigenic variability of yeast cells. During washing and mixing procedures cells were gently but thoroughly resuspended with a Pasteur pipette. Yeast cells from 3 strains of C. albicans (9938, 394, and 105) and 1 strain

of C. parapsilosis, grown to stationary phase in GYEP broth for 24 h, were washed 3 times in sterile distilled water, and 5 x 10^7 organisms (by hemacytometer count) were inoculated into 200 ml of fresh GYEPB in a 500 ml Erlenmeyer flask. Cultures were incubated at 37°C with rotation (160 rpm). At 4h, 6h, 10h, 24h, and 48h, aliquots of medium containing 10^8 cells were removed, washed 3 times in ice-cold 0.01M PBS pH 7.2, fixed in 0.25% formalin for 15 min at 4°C, reacted with 0.5 ml of mouse ascitic fluid containing monoclonal antibody H9 or C6 (agglutinin titer = 6400) or with normal mouse ascitic fluid (negative control) for 30 min at 23 to 25°C with rotation (200 rpm), and washed 3 times in 0.01M PBS. Goat anti-mouse antibody conjugated with 20 nm colloidal gold particles (40 ug/ml protein, E-Y Laboratories, Inc., San Mateo, CA) was diluted 1:2 in 0.01M PBS, and 0.1 ml was added to the yeast for 90 min at 23 to 25°C with rotation (200 rpm). Cells were washed 4 times in 0.01M PBS, prefixed in 0.5 ml of 0.25% formalin, fixed (0.5% glutaraldehyde, 1.0% acrolein, and 0.2 M sucrose in 0.075M PBS pH7.2 for 30 min at 23 to 25°C); post-fixed for 1 h in 1% OsO_4, dehydrated through a series of graded ethanol, embedded in Spurr's low viscosity resin (147), and thin sectioned. Sections were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (TEM) in a JEOL-100 CX electron microscope. A

minimum of 10 thin sections per bullet was examined from duplicate experiments and an unbiased observer evaluated sections as they were viewed in the microscope. Surface gold was quantified as 1+ (rare, < 10 particles/cell), 2+ (scant, 10-20 particles/cell), 3+ (moderate, 21-100 particles/cell) or 4+ (heavy, >100 particles/cell).

Preabsorption of diluted normal mouse ascitic fluid. Normal mouse ascitic fluid diluted 1:4 was absorbed 3 times with 10^{10} GYEPB-grown and GM-2-grown C. albicans 105 cells at 0-4°C (8 h/absorption). The absorbed fluid was subsequently diluted to the working dilution (1:10). Cells were prepared for immunoelectron microscopy as described below and results obtained when absorbed, diluted (1:10) ascitic fluid was used were compared to those obtained using unabsorbed diluted (1:10) fluid.

Pretreatment of cells with fetal bovine serum (FBS) and bovine serum albumin (BSA) to block nonspecific adherence of antibody. One ml of either undiluted FBS or 20% BSA was added to washed C. albicans 105 cells which had been grown overnight in GM-2. The mixture was refrigerated for 1 h with frequent agitation, centrifuged, and the supernatant fluid was removed and replaced with fresh FBS or BSA solution. In each case this absorption procedure was repeated 3 times and cells were examined for changes in autoagglutination and prepared for immunoelectron microscopy as described below to compare

the amount of nonspecific binding of colloidal gold-conjugated secondary antibody with that of untreated cells (cells not exposed to blocking proteins).

Trypsin treatment of germinating and nongerminating cells. One ml of trypsin solution [0.2% trypsin in Earle's balanced salt solution (BSS) pH 7.6] was added to washed cell pellets of germinating and nongerminating cells which had been harvested after 30 min, 1, 2, 4, and 24 h growth in GM-2. Control cells were exposed to the base medium without trypsin. The enzyme-cell mixtures were incubated at 37°C for 30 min with frequent mild manual agitation. After 30 min, 1.0 ml ice cold RPMI 1640 tissue culture medium containing 12% fetal bovine serum was added and cells were immediately pelleted and washed twice in cold PBS. All cells were prepared for indirect immunoelectron microscopy as described below.

Variability of surface antigens during morphogenesis. All glassware used in the preparation of germinating and nongerminating cells for electron microscopy (with the exception of flasks used to grow the organisms) were treated with silicon (Sigmacote, Sigma) to prevent germinating cells from nonspecifically adhering to glass surfaces. GM-2 was made fresh (from a 10x stock solution stored for no more than 30 d at 0-4°C) on the day of each experiment and prewarmed to 37°C prior to inoculation. C. albicans 105 was grown to stationary phase in GYEP broth.

Blastoconidia were washed in 0.01 M PBS pH 7.2 (warmed to 37°C), counted (hemacytometer), and 5 x 10^5 blastospores/ml were inoculated into 100 ml prewarmed (37°C) GM-2 in a 125 ml Erlenmeyer flask. Germination was inhibited in control flasks either by the addition of a crude extract of morphogenic autoregulatory substance (MARS) or by the inoculation of 10^8 cells/ml (52). All flasks were incubated at 37°C with rotation (160 rpm). At 0.25, 0.5, 1, 2, 4, and 20 h single flasks of germinating and nongerminating cells were removed and cells were washed in ice cold 0.01 M PBS. One ml of trypsin solution was added to all cell pellets and incubated (as described above) for 30 min. Cells were washed once in 1.0 ml RPMI 1640 with 12% FBS, twice in cold PBS, and fixed in 0.05% formalin for 15 min at 22-24°C. Each population of cells was divided among 3 tubes. Mouse ascitic fluid containing H9 and C6 (agglutinin titers = 1280) as well as normal ascitic fluid were diluted 1:10 in PBS, and 1.0 ml of diluted H9, C6, cr normal ascitic fluid was reacted with cells from each tube at each time interval for 1 h at 22-24°C with rotation (200 rpm). Cells were washed 3 times in PBS and 0.1 ml of a 1:2 dilution of goat anti-mouse immunoglobulin conjugated with 20 nm colloidal gold particles was added and reacted for 1 h at 22-24°C with rotation (160 rpm). Cells were washed 4

times in 0.01M PBS. Prefixation, fixation, postfixation, dehydration, embedding, thin-sectioning, staining, and microscopy were all done as previously described above.

Enzyme-linked antigen distribution assay (ELADA). Methods previously described (83, 84) for locating and quantifying surface antigens on red blood cells were modified for examining and enumerating surface antigens of Candida. In brief, C. albicans 9938 and S. cerevisiae were grown in GYEP broth, washed 3 times in 0.15 N saline, fixed (2.5 ml glutaraldehyde/L 0.01 M PBS pH 7.2 for 30 min), and washed in 50 mM glycine. Ten million yeast cells were reacted with monoclonal antibody (C6 or H9, multiple titers ranging from 128 to 12,800 were used); washed in PBS, and reacted with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Miles-Yeda, Rehovot, Israel). The substrate, 0.2 ml of a 0.5 g/L solution of 3-hydroxy-2-naphthoic acid 2, 4-dimethylanilide (AS-MX, Sigma) and fast blue RR salt (Sigma) in carbonate buffer pH 8.1, was added to 20 ul of the yeast cells. After 5 min cells were washed in 3 volumes of distilled water (30 sec/wash). Prepared cells were screened in a light microscope for evidence of an insoluble, purple surface complex, placed onto coverslips, allowed to air dry, sputter-coated with 10 nm of gold (Pelco Model 1, T. Pella Corp, Tuston, CA) without critical point drying (Mackler, personal

communication), and cells were examined for evidence of insoluble enzyme-substrate complexes on the cell surface in a JEOL 100CX electron microscope with an ASID-4D ultrahigh resolution (2.7 nm) scanning system at 40 kv. The experiment was attempted on 8 separate occasions. Several different formulas for carbonate buffer/substrate mixtures were used (Mackler, personal communication) in addition to several dilutions of secondary antibody (1:100, 1:500 and 1:1000). Initial cell volumes were also varied.

Background electron imaging (BEI) of colloidal gold particles. Yeast cells were prepared for BEI as described above for TEM-colloidal gold studies, except that cells were not post-fixed in OsO_4 and were coated with carbon rather than sputter-coated with gold. Secondary electron scatter was observed with a JEOL 300 scanning electron microscope equipped with a background scatter detector (at JEOL Corp., USA headquarters, Danvers, Ma.).

Characterization of antigens from crude antigen extract

Heat stability. A 5 mg/ml solution of crude antigen extract in 1 ml distilled water was heated at 3 temperatures: in a boiling water bath (96.5 ^{o}C); at 80^{o}C, and at 60^{o}C. At 10 min intervals 0.1 ml aliquots were removed and the heated extract was tested for reactivity

by Ouchterlony double diffusion with monoclonal antibodies (C6, B6, and H9).

Antigen precipitation. Three methods were used to precipitate the active fraction of the crude antigen extract. The first method was trichloroacetic acid (TCA) precipitation. Six ml of a 60% (w/v) trichloroacetic acid solution (Sigma) were added dropwise in 2 ml aliquots to a 5 mg/ml solution of the crude antigen extract. The resulting suspension was placed in an ice bath for 30 min, centrifuged (1000xg) at 5°C, and the supernatant fluid was removed from the precipitate, dialyzed against distilled water, lyophilized and rehydrated in 0.25 ml PBS. The pellet was washed in cold 20% (w/v) TCA, washed in 50% ethanol: 50% ether, and finally in 100% ether (1.0 ml each, respectively), and centrifuged. The pellet was resolubilized in 0.25 ml PBS and the supernatant fluid was allowed to evaporate at 22-24°C (under a fume hood). The residue was rehydrolyzed in 0.25 ml PBS. Rehydrated primary and secondary pellets and supernatant fluids were tested against monoclonal antibody (C6 titer = 12,800; B6 titer = 12,800; and H9 titer = 12,800) in Ouchterlony double diffusion. The second method used to precipitate the active antigen fraction was 50%-75% ammonium sulfate precipitation which was done by methods described previously (43). In the third method antigen was precipitated with cold ethanol. Fifty mg of solubilized

crude antigen in 10 ml PBS were chilled. With constant stirring, 60 ml of chilled 95% ethanol were added dropwise to the antigens in a cold room. The resulting precipitate was removed by centrifugation (300xg at 5°C), resolubilized in PBS and lyophilized. The supernatant fluid was flash evaporated at 40-50°C, resuspended in 2 ml PBS and lyophlilized. The pellet and supernatant fluids were rehydrated and tested for reactivity with the monoclonal antibodies in Ouchterlony double diffusion tests as described above.

Protein determinations. Estimations of the protein content of a 5 mg/ml crude antigen extract solution in PBS and on HPLC-isolated AgC6 and AgH9 were done by determining 260/280 ultraviolet absorption (Gilford) and using the formula (167):

$$1.54\ (OD_{280}) - 0.76\ (OD_{260}) = \text{approximate [protein]}.$$

Carbohydrate analysis of crude antigen extract. The colorimetric method of Dubois et.al. (36, H_2SO_4-phenol method) was used to determine the carbohydrate concentration in crude antigen extracts.

Gel electrophoresis of crude antigen extract. Polyacrylamide gels, under reducing and nonreducing conditions, were run on crude antigen extract by Laemmli methodology (74). Ten percent gels were run with 5% spacer gels. Gels were loaded with protein concentrations varying from