Structural determination and function of a cell wall epitope of Candida albicans
by Ren-Kai Li

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University
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Abstract:
An IgM monoclonal antibody was derived which agglutinates Candida albicans. The expression of the corresponding antigen is variable during growth of the yeast and mycelial forms of the fungus. Antigen expression was investigated at the ultrastructural level by transmission electron microscopy and immunolabeling with colloidal gold-conjugated antibodies. Antigen is mainly located on the plasma membrane of logarithmic phase yeasts, and becomes dense on the cell wall surface of stationary phase yeasts. In germinating hyphae, although antigen is expressed primarily on the surface of parent cells, expression on developing hyphae is growth phase dependent. The antigen isolated from yeast cell wall by immunoprecipitation was partially characterized. The epitope which is recognized by the monoclonal antibody was isolated and purified from the cell wall extract and structurally determined by gas chromatography, mass spectroscopy, and nuclear magnetic resonance. The epitope contains a linear mannotetraose with β-1,2-glycosidic linkages. Both the epitope and the immunoprecipitated antigen have the ability to inhibit attachment of C. albicans to mice spleen tissue. The antigen recognized by the monoclonal antibody is one of the adhesin molecules involved in the attachment of C. albicans to mouse spleen marginal zone macrophages. The epitope was identified as an adhesion site on C. albicans for this tissue-specific adherence.

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committee and has been found to be satisfactory regarding
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College of Graduate Studies.

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ABSTRACT

An IgM monoclonal antibody was derived which agglutinates *Candida albicans*. The expression of the corresponding antigen is variable during growth of the yeast and mycelial forms of the fungus. Antigen expression was investigated at the ultrastructural level by transmission electron microscopy and immunolabeling with colloidal gold-conjugated antibodies. Antigen is mainly located on the plasma membrane of logarithmic phase yeasts, and becomes dense on the cell wall surface of stationary phase yeasts. In germinating hyphae, although antigen is expressed primarily on the surface of parent cells, expression on developing hyphae is growth phase dependent. The antigen isolated from yeast cell wall by immunoprecipitation was partially characterized. The epitope which is recognized by the monoclonal antibody was isolated and purified from the cell wall extract and structurally determined by gas chromatography, mass spectroscopy, and nuclear magnetic resonance. The epitope contains a linear mannotetraose with β-1,2-glycosidic linkages. Both the epitope and the immunoprecipitated antigen have the ability to inhibit attachment of *C. albicans* to mice spleen tissue. The antigen recognized by the monoclonal antibody is one of the adhesin molecules involved in the attachment of *C. albicans* to mouse spleen marginal zone macrophages. The epitope was identified as an adhesion site on *C. albicans* for this tissue-specific adherence.

Chapter 1

INTRODUCTION

Candida albicans is frequently encountered as a normal commensal of the digestive and genitourinary tracts of human and various warm-blooded animals. As an opportunistic pathogen, *C. albicans* is the major etiological agent of candidiasis, a disease with conditions that range from superficial infection to systemic and multi-organ (disseminated) infection. Other species, including *C. stellatoidea, C. tropicalis, C. parapsilosis, C. krusei, C. guilliermondii, C. glabrata, C. lusitaniae,* and *C. pseudotropicalis*, although less frequently encountered, may also cause various clinical forms of candidiasis (254).

Cell wall components are intimately involved in the initial contact of *C. albicans* with its hosts. The biochemical characteristics of the cell wall of *C. albicans*, which underlines its virulence potential, is a very complex issue. This complexity is magnified by *C. albicans'* ability to undergo rapid topographical changes depending on the environmental conditions. Antigenic variation and dynamic expression of mannoprotein antigens (26,27,30) and surface hydrophobic molecules (125) during morphogenesis have been

documented. These surface changes could in turn influence the pathogenesis and efficiency of host defense. Determination of the structure and function of cell wall components is essential for understanding the host-*C. albicans* relationship.

My dissertation has focused on the structural determination of a cell wall antigen of *C. albicans* and its role in host-*C. albicans* interaction. An enormous literature has accumulated in the past few years on various aspects of *C. albicans* and the disease, candidiasis, caused by this fungus. This introduction will be limited to topics related to my research, which includes a brief consideration of conditions that predispose the host to candidiasis, approaches used to diagnose disseminated candidiasis, host defense mechanisms, proposed virulence factors of the fungus, and a review of cell wall components and their interactions with the host.

Factors Predisposing to Candidal Infections

In general, diseases or treatments of disease which create immunosuppressive conditions are likely to predispose to candidal infections. Patients suffering from acquired immunodeficiency syndrome (AIDS) are among those at high risk for candidiasis. In fact, mucosal involvement with *Candida* is so common in AIDS patients that oral candidiasis is one of the criteria established by the Centers for Disease Control

(CDC) for identifying individuals at risk for the development of AIDS (1,261).

With increasing therapeutic modalities available for medical care, the incidence of disseminated candidiasis has increased markedly in the past few decades (81,167,233,247,353,359). Systemic candidiasis has become an important cause of morbidity and mortality in patients receiving aggressive antineoplastic chemotherapy and recipients of bone marrow transplants. There are many other conditions that promote the transition of *C. albicans* from an innocuous resident of the mucosal surfaces to a harmful invader. Treatment with antibiotics, especially broad-spectrum or multiple narrow-spectrum antibiotics, upsets the ecological balance of the gut and promotes overgrowth of the fungus. The use of intravascular catheters predisposes to candidemia and systemic forms of disease (3). Patients suffering from neutropenia are at high risk for disseminated candidiasis (246). Other factors that have been reported to predispose to candidiasis include pregnancy, old age, and trauma (183,254). Endocrine disorders have also been reported to predispose to candidiasis (2,246,285). Diabetes mellitus predisposes to candidiasis and other infections. High glucose levels in the blood and other tissues of diabetic patients may explain in part the increased host susceptibility to candidiasis. Knight and Fletcher suggested that high glucose concentration favors the growth of Candida

(167). Wilson and Reeves reported that, in diabetics, the concentration of NADPH in neutrophils is reduced by the conversion process of glucose to sorbitol, and thus lowering the amount of NADPH available for the oxidative killing mechanisms of neutrophils (363).

Diagnosis of Candidiasis

Several methods, including histopathologic examination of patient biopsy material, specimen culture and numerous serological tests, have been described that facilitate the diagnosis of candidal infections (1,61,97,99,196,321,332). Despite the fact that *C. albicans* has received a great deal of clinical and research investigation, the diagnosis of disseminated forms of candidiasis is still problematic. In one study, blood culturing, a routine procedure in clinical laboratories, showed only a 44% positive result of patients with autopsy-proven candidiasis (247). Furthermore, in an experimental animal model, invasive candidiasis often showed low levels of fungemia (139). Conversely, candidemia does not always lead to systemic disease (356), however, multiple positive cultures correlates well with systemic candidal infections (114). In other body sites containing a microbiotic flora, such as the respiratory, digestive, and urinary tracts, the isolation of *C. albicans* is difficult to interpret, especially if the patient has predisposing factors for a high rate of colonization (183,196).

One approach for the serodiagnosis of systemic candidiasis is to detect antibodies against the cell wall or cytoplasmic components of *C. albicans* (149,225,230,244). Various immunochemical methods including immunofluorescence, immunoelectrophoresis, double diffusion, radioimmunoassay, and enzyme-linked immunosorbent assays have been tried. The sensitivity of these assays ranged from 50 to 92% in immunocompetent patients, but less sensitivity in immunosuppressed patients, which is the population at greatest risk (101). This limitation is amplified by the fact that serodiagnostic assays do not distinguish colonization and superficial infection from invasive disease because the majority of apparently healthy individuals have antibodies to *Candida* cell wall mannans (79,114,196).

Theoretically, the presence of serum antibodies to *C. albicans* cytoplasmic components should be more indicative of systemic infection than antibodies to cell wall components. It was postulated that exposure to cytoplasmic antigens of *C. albicans* should not occur unless fungal elements in tissue are ingested by phagocytes, which then release *C. albicans* cytoplasmic antigens (332). However, measurement of antibodies to cytoplasmic antigens has also failed to distinguish between colonization and invasive infection (133,225) probably because of cross-reactivity of such antibodies with cell wall mannans (328). One of the detection systems based on this strategy was reported to

differentiate between *C. albicans* colonization and systemic infection, but the sensitivity was low (97).

Another serodiagnostic approach is to detect candidal antigens or metabolites in patients' serum or tissue (6,60,102,158,159,232,322,355). These methods have a common advantage in that the results are not dependent on the ability of the patient to mount a humoral immune response. Fungal-specific mannan is one of the most abundant antigen on the surface of *Candida* cells and was thus considered as an indicator for systemic infection (238). In several studies, mannan detection demonstrates specificity of above 90%, with very few patients experiencing colonization or superficial infection showing antigenemia (20,61). However, in many infected patients, the concentration of circulating mannan is below the limit of detection of assay techniques, such as radioimmunoassay (RIA) or enzyme immunoassay (EIA) (197). In addition, antigenemia may occur only intermittently, requiring that multiple serum samples be taken during the period of risk (92,149,197). The assay reliability may also be affected by different serotypes of the infecting strain of *C. albicans* (see below) because serotype B mannan is less reactive to serotype A reagent (279). The clinical application of this approach would largely depend on the development of reagents for various *C. albicans* strains and the enhancement of detection sensitivity.

Another approach is to detect metabolic products of *C.*

albicans. D-arabinitol, a five-carbon, unbranched alditol, was reported in the serum of patients with systemic candidiasis and was suggested as a detecting target for the diagnosis of disseminated candidiasis (60,159,283). However, there are problems associated with this diagnostic strategy because of increased serum arabinitol concentrations in patients with renal dysfunction, and the absolute values and variability of endogenous arabinitol. An approach to the first problem was the use of arabinitol/creatine ratios rather than just arabinitol concentration (109). In a study of 25 patients, the detection of arabinitol in serum by gas-liquid chromatography, and subsequent correction for renal insufficiency by calculation of the arabinitol /creatinine ratio yielded a sensitivity of 64% and a specificity of 96% (109).

The answer to the second problem indicated above comes from the understanding that the arabinitol produced by *C. albicans* grown in serum is of the D configuration (159,283). While in human there are both enantiomers of arabinitol, endogenous serum and urine concentrations of both D- and L-arabinitol is a rather fixed ratio in normal individuals (284). Renal dysfunction without candidiasis also yielded normal D/L ratios despite high arabinitol concentrations (284). It was suggested that the measurement of D/L arabinitol ratios by gas chromatography, without even determining the concentration of the separated enantiomers,

is adequate for the differentiation of arabinitol between fungal and non-fungal origins (284).

However, because of its complexity, the assay method is not very practical for most clinical laboratories. The separation and identification of D- versus L-arabinitol require internal standardization, preparation of volatile derivatives, and unconventional facilities for gas chromatography and mass spectrometry.

Monoclonal antibodies produced against the cytoplasmic components revealed an immunodominant protein antigen of 48 kilodalton (kd) (321,322). From the deduced amino acid sequence of the cDNA clone, the 48 kd antigen shows high homology to the glycolytic enzyme enolase (220). Several other groups have also identified an antigen of C. albicans in the 44-60 kd size range (7,113,223). It was suggested that all these antigens are probably one and the same (90). The immunodominant antigen can be detected in patients with disseminated candidiasis (113,321). A report on the immunoassay detection of the circulating enolase-like 48 kd antigen in high-risk patients showed an 85% sensitivity for deep tissue infection and 64% sensitivity for fungemia (352). This method appears to be complementary with the blood culture test as half of the tissue-proved cases were recognized by both methods, the remaining half were recognized by one system or the other alone (352).

Other laboratories have also reported detection of

protein antigens of cytoplasmic origin in patients with invasive candidiasis (6,102). The application of latex agglutination using latex beads coated with antibodies from immune rabbit serum may be used in combination with other serological tests to facilitated the detection procedures (102,270).

So far, the laboratory tools for the diagnosis of systemic candidiasis are limited. Blood cultures are slow, insensitive, and non-specific for fungemia and disseminated infections. Detection of antibodies to *C. albicans* is also unable to discriminate superficial from systemic infections, and not helpful in immunosuppressed patients. Detection of fungal products can be specific, but the sensitivity of current assays is not satisfactory. Commercially available latex agglutination kits lack either specificity or sensitivity. The application of possible DNA probes for diagnosis has not been productive although some *C. albicans* specific DNA fragments have been isolated (58,211,221,237,297). These obstacles may be overcomed by the combination of various assay methods, however, further improvement of the diagnostic techniques will be based upon an in depth understanding of the fungus, especially on its pathogenic mechanisms and its interactions with its hosts.

Host Immunity

Under normal circumstances, host innate factors, such as

the intact skin and mucous membranes, normal flora, phagocytosis and complement system, mediate the primary protection against fungal infections (246,367). The importance of polymorphonuclear neutrophils (PMN) and monocytes in defense against disseminated candidiasis has been documented (10,62,64,78,80,140,141,191). Individuals with defective PMN functions or neutropenia are at high risk of disseminated candidiasis (246). Several candidacidal mechanisms have been defined in human PMN such as the myeloperoxidase (MPO)-dependent oxidative burst (62,191). Neutrophils from MPO-deficient patients possess normal phagocytic ability, but their ability to kill ingested *Candida* cells is much lower than that of the normal PMN. Receptor-mediated recognition and killing of non-engulfed *C. albicans* pseudohyphae by human PMN has also been reported (205,226). Evidence also suggests important alternative fungicidal pathways that are MPO-independent (190,192,193).

The ability of PMN or other phagocytic cells to ingest *Candida* cells does not necessarily mean an effective killing of the organism (19,143,202,319). Leijh et al.(194) reported that up to 50% of the ingested *C. albicans* remained viable after one hour of incubation. Louria and Brayton (202) found that although mouse PMN were able to ingest up to seven *C. albicans* cells in 30 minutes, 64% of these PMN had pseudohyphae penetrating their cell membranes after 4 hours of incubation. Other investigators showed that the

phagocytic killing of *C. albicans* by phagocytic cells is regulated by complement factors (100,179,239,362,367) or factors secreted by other cell types upon incubation with *C. albicans* (64,65).

Mechanisms against *Candida* infections are also believed to involve specific T cells. Individuals with depressed cellular immune systems are particularly susceptible to mucosal candidiasis, and patients with chronic mucocutaneous candidiasis often have defective delayed-type hypersensitivity (DTH) reactions to *Candida* antigens (163,242,265,347,349). However, patients with defects in cell-mediated immunity rarely develop disseminated forms of candidiasis (254). In addition, passive transfer of *C. albicans*-sensitized lymphoid cells failed to protect against disseminated candidiasis, although the cells transferred DTH reactivity to candidal antigen in recipient animals (260). The protective role of cell-mediated immunity in systemic candidiasis is not well defined (54,141,286), however, recent work suggest that T-cell immunity contributes to host defense by the production of various cytokines that activate PMN candidacidal functions (42).

Other cell populations that may be important in defense against systemic infection of *C. albicans* include natural-killer cells (NK cells) and lymphokine-activated killer cells (LAK). In the majority of studies, NK cells do not appear to directly kill *C. albicans*, but through the secretion of

cytokines, they cause activation of phagocytic killing abilities (64,65,215). LAK cells are derived from the large granular lymphocytic, natural killer subset of lymphocytes (354). These cells have cytotoxic reactivity against a variety of tumor cell lines (8,295). LAK cells from murine spleen has also been reported to acquire the capacity to inhibit the growth of *C. albicans* (21). Recently, the ability of LAK cells to down-regulate monocyte-mediated antigen presentation has been shown (354). In this report, both antimicrobial activity and antigen presenting function of monocytes are susceptible to inhibition by LAK cells. It was speculated that the regulation may represent a system developed to specifically suppress accessory cells at certain stages of activation for the control of immune homeostasis (354).

Serum factors have also been suggested as one of the initial host defense mechanisms against *Candida* infections. A serum factor (neither complement nor immunoglobulin) has been described that exerts an inhibitory effect of *C. albicans* by causing yeast clumping (203,311). Complement system appears to increase the phagocytic killing ability of PMN and monocytes by opsonization of *C. albicans* (100,241,367). Components of *C. albicans* cell wall capable of activating the alternative complement pathway have been reported (179,276,334). Recently, receptors on *C. albicans* for complement fragments (iC3b, C3d) have been characterized

(34,93,152). Postulated roles for such molecules as a virulence factor will be discussed.

The functional role of humoral immunity in protection against systemic candidiasis is not clear. Normal persons often carry antibodies to *C. albicans* in their serum due to alimentary tract colonization (117,163,254). Patients with candidiasis typically manifest normal (or even higher) levels of agglutinins and precipitins against *Candida* antigens (164). Some investigators suggest that antibody (induced or passively transferred) has little or no effect on the protection against systemic candidiasis (11,16,148). However, there are reports on the capacity of immune serum to passively transfer resistance to *C. albicans* infection in experimental animals (148,223,243,260). Mourad and Friedman (243) have demonstrated that subcutaneous injection of mouse anti-*Candida* antiserum significantly protected animals from intravenous *C. albicans* challenge. Similar results were also reported by Pearsall *et al* (260). Matthews *et al.*(222,223) presented evidence suggesting that antibody to an immunodominant 47 kd candidal antigen may protect against invasive candidiasis. This 47 kd antigen has recently been shown to be a breakdown product of *C. albicans* heat-shock protein 90 (hsp 90) which shares conserved epitopes with human hsp 90 (224). It was reported that all patients recovering from systemic infection of *C. albicans* produced antibodies against conserved epitopes on both fungal and

human hsp 90, and that autoantibodies to hsp 90 could mediate protection against systemic candidiasis (224).

In summary, it is generally accepted that PMN are the major defense against systemic candidiasis. However, since products of lymphocytes, such as interleukin-2, can influence circulating phagocytes, it is difficult to separate the innate defense system from the acquired immune mechanism. In addition, the investigation of host immunity against *C. albicans* is further complicated by the immunomodulative potential of *C. albicans* cell wall materials (40,67,259,262,295) (discussed below). As mentioned earlier, multiple cell populations (e.g., NK cells and LAK cells) as well as serum factors (e.g., complement, cytokines, antibodies) have been implicated in the immune response to systemic fungal infections and the relative contribution of each factor in protection against specific forms of candidiasis remains to be elucidated [reviewed by (112,163,246,285)].

C. albicans Virulence Factors

An enormous amount of information and a large number of review articles on the topic of host-*C. albicans* interactions have accumulated in recent years. Aside from the individual susceptibility difference of the host, many biochemical properties associated with the cell surface of *C. albicans* have been attributed to the virulence of this fungus. These

include the secretion of acid proteinase
(75,103,177,184,208), morphological changes (218,313),
recognition and adhesion to host tissue
(34,56,107,151,293,308), cell surface hydrophobicity (5,156),
toxin production (57,126,290,291) and immunoregulatory
activity of cell wall materials (40,59,66,67,98,117,263). It
is likely that *C. albicans* virulence is a function of a
multiplicity of factors rather than one single determinant.
Some general information of these host-*C. albicans*
relationships will be described.

<u>Morphogenesis.</u>

An intriguing characteristic of *C. albicans* is its
polymorphism. Depending on the environmental conditions, *C.
albicans* can exist as ovoid, budding yeasts, as mold-like
hyphal/mycelia with septa, as intermediate-form pseudohyphae,
or as chlamydospores (48,209,254,255,264,365). Unlike other
dimorphic fungal pathogens, such as *Coccidioides immitis*,
Blastomyces dermatitidis, and *Histoplasma capsulatum* in which
the tissue phase is exclusively that of the yeast form, *C.
albicans* in infected human tissues usually consists of a
mixture of budding yeasts, hyphae, and pseudohyphae (254).
For this reason, the ability of *C. albicans* to grow
polymorphically has generally been accepted as a virulence
trait and used as an indicator for histopathological
identification (254,282). In fact, a routine procedure for

identification of *C. albicans* is the germ tube test. Other *Candida* species, except for *C. stellatoidea* and rare isolates of *C. tropicalis*, make only pseudohyphae with species-distinctive morphology (254,255).

The ability of yeast cells to germinate and produce hyphae is a characteristic which may be a virulence factor of *C. albicans* (55,289). It is a general assumption that hyphae penetrate tissue more readily than yeast cells, and hyphae are more difficult to ingest. However, definitive evidence supporting this assumption is lacking, and the results from various laboratories are controversial (218,289,312,364). Hyphae have been suggested to be capable of eluding phagocytosis by penetrating the cell membrane of phagocytes (202). On the other hand, Diamond *et al* (62,204) produced *in vitro* evidence that engulfment is not required for the killing of hyphal element by PMN.

By using mutant strains that produce either yeasts or mycelia, Shepherd (300) reported that both morphological forms are capable of invading soft tissue and causing systemic infection in mice. Conclusions from this study are limited, however, for at least two reasons. First, other possible genetic defects associated with the morphological mutation were not accounted for. Second, the inoculum of mycelia was on a dry weight basis which is not likely to be comparable to cell counts of the yeast inoculum (55). In fact, it is difficult to draw a correlation between the

pathogenesis and the morphological forms considering that the genetic and biochemical mechanisms of *C. albicans* morphogenesis is not clearly understood (255).

Adherence.

The ability of *C. albicans* to adhere to tissue or other surface has been implicated for its pathogenicity (18,103,150,162,228,298). It was shown that *C. albicans* and another *Candida* species that can cause disease, *C. tropicalis*, adhere to host cells to a greater extent than other relatively nonpathogenic species such as *C. krusei* and *C. guillermondii* (162). Segal *et at* (298) reported that *C. albicans* isolates from patients with vaginitis were significantly more adherent to human vaginal epithelial cells than isolates from asymptomatic carriers. However, there was no direct correlation between virulence and adherence ability when various strains of *C. albicans* were tested (154).

Hyphae are also more adhesive to epithelial cells than are yeast cells (160,161,293,313). Incubation of *C. albicans* under conditions favorable for germination increased its adherence to buccal epithelial cells (161). It was recently reported that different adhesins are involved in the adhesion of yeasts or germ tubes to human endothelial cells (77). Whether the enhanced adherence ability is due to degradation or movement and reorganization of the cell wall materials is not clear (55).

Mechanisms involved in the adherence of *C. albicans* to host tissue have been proposed and several adhesin molecules have been suggested [reviewed by (33,34,55,69)]. The ability of *C. albicans* to bind complement components (C3d, iC3b) and extracellular matrix proteins (laminin, fibronectin, and collagens) has been suggested as an adhesion and virulence factor (34,55,69,152,166). Lectin-ligand molecules have also been reported in which the adhesion can be blocked by various sugars (49,69). In addition, a serotype A-specific oligosaccharide (factor 6) isolated from *C. albicans* cell wall was found to be largely involved in the adhesion of the fungus to human buccal epithelial cells (236,326). Ultrastructural observation revealed an outer fibrillar-floccular layer on the cell surface of *C. albicans* (29,267,337). This layer has been shown to be mannoproteins, and the appearance of this layer on the cell surface correlates with the adherence ability of *C. albicans* (50,70,228,229,342). Although most of the evidence indicates a role for cell wall mannoproteins in mediating the attachment (50,70,229,293,314), the specific adhesion molecule(s) on *C. albicans* and its ligand on host tissue has not yet been identified.

Hydrophobic interactions between *C. albicans* cell surface protein(s) and molecule(s) of the host have also been suggested to be one of the adhesion mechanisms and virulence factors (56,120). *C. albicans* yeasts grown at room

temperature are more hydrophobic than yeasts grown at 37°C (118). Hydrophobic yeasts are more virulent than hydrophilic yeast cells in a mouse model (5). The significant correlation between the expression of cell surface hydrophobicity (CSH) and its adherence to HeLa cells has been documented (119). Different binding patterns of hydrophobic and hydrophilic cells on sections of mice spleens and lymph nodes were also noted (56,121). Hydrophobic yeasts bind throughout the sections while hydrophilic yeasts bind specifically to regions rich in macrophages. It was reported that hydrophobic molecules are proteins and that mannosylation may influence the exposure of these surface hydrophobic proteins (125).

It is interesting that CSH also correlates with the morphological changes of *C. albicans*. Hydrophobic cells germinate more quickly than hydrophilic yeasts, and the increase in CSH is reported as an event that precedes germination (118). This may partially explain the greater adherence of hyphae to epithelial cells as mentioned above. Thus, it is possible that CSH contributes to the pathogenicity of *C. albicans* through multiple mechanisms which include enhanced adherence and hyphal-ready characteristics (55).

Enzymes.

Secretion of enzymes, especially proteinases and

phospholipases, has received great attention for its possible correlation with the pathogenesis of *C. albicans*. *C. albicans* and some other *Candida* species secrete proteinases when they are grown in media containing proteins as the sole nitrogen source (207,287). Staib's group (280) first purified the extracellular proteinase and reported that the ability of *C. albicans* to use serum proteins as a source of nitrogen is related to relative strain pathogenicity (317,318). The pathogenic role of *C. albicans* proteinase has been reviewed (33,34,55,104,278), and several mechanisms were proposed. Except for its proteolytic activity which may aid tissue invasion (277), Borg and Ruchel (23) reported that the adherence of *C. albicans* to human buccal epithelial was significantly reduced when the proteinase inhibitor, pepstatin A, was added to the incubation mixture. Thus, *Candida* proteinase may be involved in fungal adherence by modification of surface molecules (55,278).

The Cell Wall of *C. albicans*

Most of the knowledge about the structure and biosynthesis of fungal cell wall polysaccharides is from studies on Baker's yeast (*Saccharomyces cerevisiae*). However, in the last few years a great deal of attention has been focused on the cell wall of *C. albicans*. The cell wall of *C. albicans* is composed predominantly of β-glucans, mannoproteins and chitin (82,85,288). Various techniques

including transmission electron microscopy, protoplast regeneration, and several cytochemical staining techniques have revealed chemically-distinct layers in the cell wall (178,267,269,271,273,288). In yeast-form cells, mannoproteins are mostly found on the outermost and inner layers while chitin spans the inner regions of the cell wall, septa, and bud scars (14,32,86,89,136). It was reported that the content of chitin increased at least three-fold during germination (45). More recent evidence suggests that in the mycelial form of *C. albicans*, mannoproteins are distributed throughout the entire cell wall structure and some of the mannoproteins are covalently linked to chitin (214).

Glucans (D-glucose linked in the β-configuration) account for about 60% of cell wall dry weight (89,301). Since glucans have not been found to be immunogenic, it is unlikely that they play a role in elicitation of specific immune responses (66). However, glucans from *C. albicans* and other fungi have been shown to have endotoxin-like activities (131). Data also suggest that glucan is able to modulate non-specific immune responses of the host presumably through the activation of PMN or NK cells (176,294,360).

Alteration in glucan content during growth and germination were also reported (110). It was proposed that the *C. albicans* cell wall contains at least three types of glucans depending on their linkages and acid or alkaline solubility (110). During protoplast regeneration and early

stages of germination, glucan synthesis is almost exclusively of the alkaline-insoluble β-1,3-glucan (110,180), while the synthesis of β-1,6-glucan appears to contribute to the secondary wall layer (41). Part of the alkaline-insoluble glucans are linked to chitin and provide the rigid framework of the cell wall (83,84). Evidence has been provided that the linkage between the two components of the cell wall does not involve peptides but occurs directly through acetylglucosamine and glucose via β-1,6-linkages (306,307). It has been suggested that a large portion of mannoproteins are covalently linked to β-glucans, and therefore reagents such as dithiothreitol (DTT) and sodium dodecyl sulfate (SDS) are not able to solubilize all of the wall mannoproteins (369). Hydrolysis of the β-1,3-glucan by the action of β-1,3-glucanase (Zymolyase) is required for cell wall dissolution and extraction of all mannoproteins. Data from protoplast regeneration experiments indicate that inhibition of glucan synthesis by papulocandin B prevents incorporation of glucanase-extractable mannoproteins into the cell wall while the incorporation of SDS-extractable mannoproteins are not affected (84,245). However, the type of bond(s) linking mannan or proteins to glucans is not known.

The chemical composition and function of *C. albicans* cell walls compares favorably with that of *S. cerevisiae* (40,86,89,180,234). However, significant differences in the content of chitin and mannoproteins in these two species have

been reported (84,127,180,256,305,350,351).

Mannoproteins of yeasts have been categorized into functional, such as invertase and acid proteinase, and structural mannoproteins which constitute intrinsic components of the cell wall (13). The mannose-rich polysaccharides of the mannoprotein appear to have been conserved through evolution, as evidenced by the resemblance of yeast mannoprotein core oligosaccharides to carbohydrate fragments derived from human IgM (43,44,248). Similar oligosaccharides also occur in glycoproteins of other fungi such as α-amylase from *Aspergillus oryzae* (366), in hen ovalbumin (329), and in calf thyroglobulin (144).

Mannan (mannoproteins) in yeasts account for a major part of the carbohydrate content and immunogenicity of the *C. albicans* cell wall. As the name implies, the principle carbohydrate in mannan is D-mannose, however, other sugars including D-glucose, D-galactose, D-xylose, L-arabinose, L-rhamnose, L-fucose and D-glucuronic acid were also found in yeast mannans (14,89). Chemical analysis has shown that *C. albicans* mannan contains highly branched polysaccharides in which mannose units are joined via α-1,6-linkages to form the main chain with side chains of various length attached via α-1,2- and α-1,3-linkages (Figure 1) (14,368). The size and number of these side chains are species- and strain-specific (288). The main chains are linked to proteins through di-N-acetylchitobiose (N-acetylglucosamine dimer) to asparagine

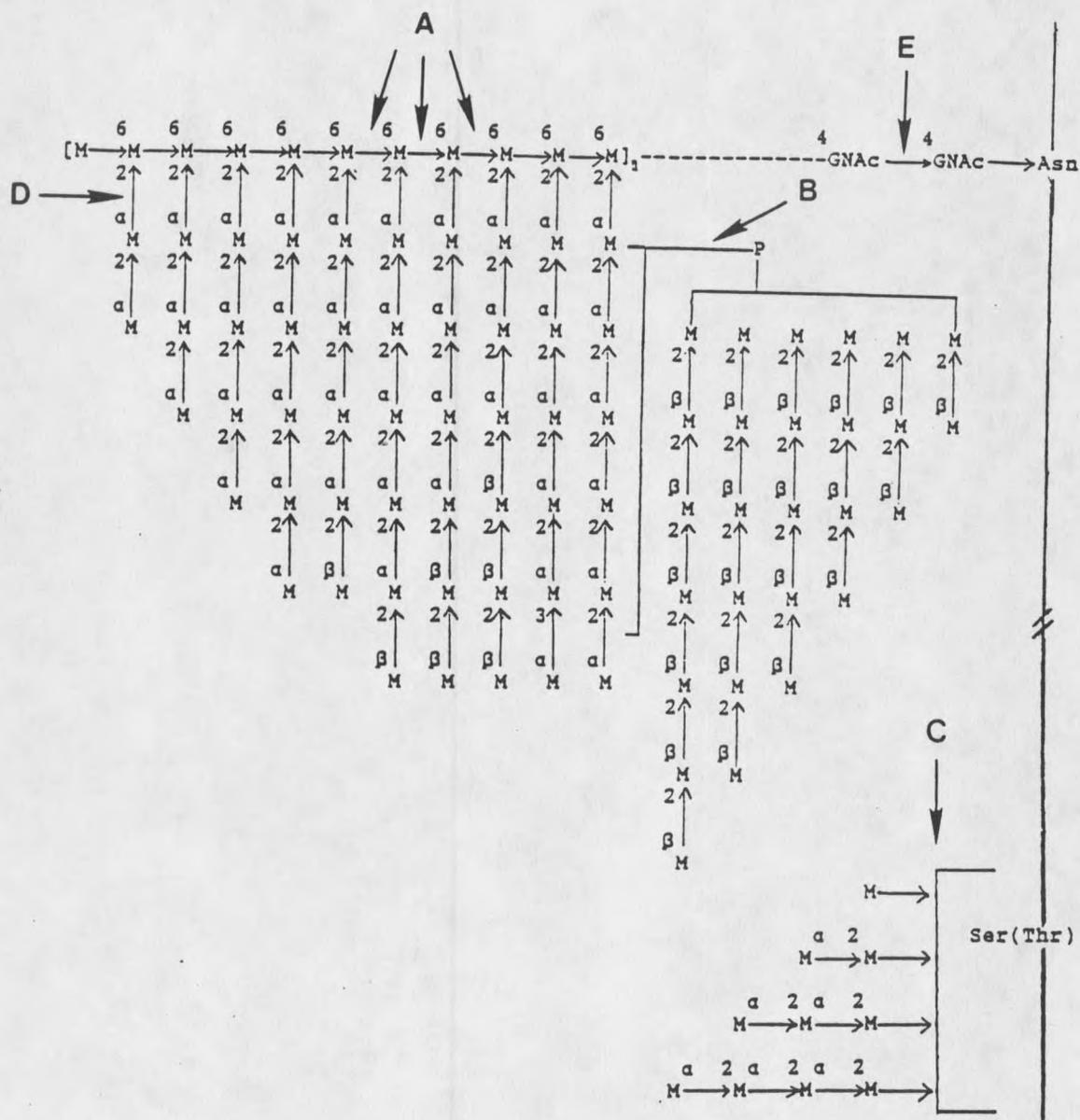**Figure 1.** Proposed cell wall mannan structure of *C. albicans*. Terminal β-linkages in the side chains are serotype A-specific. M, D-mannose. GNAc, N-acetylglucosamine. P, phosphate group. Arrows A, B, and C are cleavage sites of mild acetolysis, mild acid hydrolysis, and alkaline degradation, respectively. Arrows D and E indicate cleavage sites of exo-α-mannanase and Endo-β-N-acetylglucosaminidase (Endo-H) treatment, respectively.

(N-linked) (Figure 1). The biosynthesis of N-linked oligosaccharides in yeasts and animal glycoproteins are similar in that both begin with the transfer of a glucosylated mannose-rich fragment from dolichol pyrophosphate to the proteins (181,187,258,344). Some of the mannosyl oligosaccharides are linked through homologous α-1,2-linkages and attached to proteins via O-glycosidic linkages to the hydroxy amino acids threonine or serine (O-linked) (Figure 1) (82,117,331). In *C. albicans* mannoproteins, over 85% of the mannan is N-linked and less than 15% is linked O-glycosidically to proteins (82,89).

The N-linked mannan of *C. albicans* contains phosphate groups that link mannosyl residues via a phosphodiester bond to a variable number of side chains of oligomannose units. The high content of phosphate in the *C. albicans* cell wall makes it resistant to mannosidase digestion (145). The phosphodiester bonds appear to be the most acid-labile linkages in the mannoprotein complex (14). Methylation and proton nuclear magnetic resonance (^{1}H NMR) analysis of mild acid-hydrolyzed fractions indicate that the phospho-oligomannosyl contains homologous β-1,2-linkage (Figure 1) (170,171,303). It was proposed that the phosphomannosyl side chains display the major immunogenic epitopes that are responsible for antibody production of the host (303-305).

Various chemical and biochemical methods have been applied to the study of yeast cell wall mannans. As

indicated in Figure 1, certain acetolysis conditions split 1,6-linkages in preference to other glycosidic bonds, and convert many yeast mannans to a mixture of small oligosaccharides with intact side chains (174,320). Mild acid (10 mM HCl) hydrolysis cleaves the acid-labile phosphodiester linkage (327) while alkaline treatment (0.1 M NaOH) causes β-elimination and cleaves the O-linked polymannose from the protein moiety (Figure 1) (15,22,240). Enzymes such as Endo-H, α- or β-D-mannosidase, and exo-α-mannanase are also useful for characterization of polysaccharides from yeast mannans (145,248,331,341). Exo-α-mannanase hydrolyses manno-oligosaccharides with α-1,2- and α-1,3-linkages, leaving the backbone essentially intact (Figure 1) (146). Combinations of these methods with size exclusion chromatography and other techniques have provided a powerful means for structural analysis of mannans (12,275,304).

Studies of mannoproteins from a number of different isolates of *C. albicans* indicate that two serotypes, A and B, exist. The serotype A strains of *C. albicans* spp. and *C. tropicalis* spp. share identical antigens, and the serotype B strains of *C. albicans* spp. and *C. stellatoidea* spp. share identical antigens (115,116). Antiserum against A strains agglutinate strain A after it has been absorbed by strain B, while antiserum against B strains does not agglutinate strain B after heterogenous absorption by strain A. These results

suggest that serotype A strains contain all antigenic determinants of serotype B plus additional determinants produced only by serotype A strains (115). Sunayama and Suzuki (323) suggested that the immunochemical differences in the mannans of *C. albicans* serotype A and B reside at least in differences in the length of the side chains which have α-1,2-linkages. Later, Fukazawa *et al* (94) demonstrated that *C. albicans* serotype A possesses antigenic factor 6 while serotype B has factor 13b instead of 6, and that antigenic factor 6 is important for the identification of serotype A strains.

Many studies of the serotype-specific epitope have been reported and the results are controversial (95,106,138,147,169). One of the mannohexaose fragments obtained from acetolysis, which consisted of one terminal α-1,3-linkage in addition to four α-1,2-linkages, was reported by Fukazawa *et al* to be responsible for the specificity of antigenic factor 6 (326). However, the same structure was also found in mannan from serotype B strains as reported by Kobayashi *et al* (171). These authors proposed non-reducing terminal β-1,2-linkages in one of the serotype A-specific epitopes (303). The relevance of β-linkages in serotype A-specific determinants was substantiated by the finding that when serotype A yeasts were grown at pH 2.0, the absence of β-linkages and phosphate groups in the cell wall correlated with the loss of agglutination with factor 6 serum (168).

Both serotypes contain phosphomannan of the same structure (169), thus it is unlikely that serotype specificity lays in the homologous β-linked phosphomannan (303). However, Shibata et al have reported that the β-linkage of serotype B exists only in the acid-labile region connected by a phosphate group to the side chains (Figure 1). In the mannan of serotype A, two types of β-linkages are found; one located in the acid-stable, and the other in the acid-labile regions (Figure 1) (304). The β-1,2-linkages in the acid-stable domain are also found in C. stellatoidea type II and C. glabrata, the cells which are known to be agglutinated with factor 6 serum (172,236). Recent studies on precipitin inhibition with structurally defined oligosaccharides show that epitopes containing three α-1,2-linkages in addition to one, two, or three β-1,2-linkages on the non-reducing terminals participate in the serotype A specificity of C. albicans strains (172).

Although the total mannan content did not vary greatly with the stage of growth or germ tube formation (301), structural variations in the cell wall mannan during morphogenesis has been reported. A remarkable suppression of mannan biosynthesis takes place during yeast to mycelium transformation, especially the synthesis of β-1,2- and α-1,3-linked mannopyranosyl residues which account for the side chains and phosphomannan. It was suggested that the decrease in mannan synthesis results in less complete structure and

less specific antigenicity in antibody responses (303,305).
In this context the presence of germ tube-specific antigens
is of particular interest (38,39,257,310). Epitopes specific
for the hyphal phase of *C. albicans* were mostly found in the
mannoproteins (38,257,310,324,325) whereas epitopes common to
blastoconidia and hyphae were stable to heat treatment,
reducing agents, and proteolytic enzymes (134,324). Other
than *de novo* synthesis of new proteins during germination,
the possibility exists that the detection of germ tube-
specific protein antigens reflects the differences in the
carbohydrate moiety of mannoproteins of the two growth phases
(38).

The outermost layer of the cell wall of yeast cells has
been shown to contain mannoproteins which form a mosaic of
antigenic determinants (28,41,268). Aside from its possible
role in adherence to host tissues, the pathogenic potential
of cell wall mannoproteins is suggested by its ability to
modulate immune responses in both human and experimental
animals (59,68,215).

Two types of immunomodulation (activation or depression)
have been observed in experimental mice upon injection of *C.
albicans* preparations, depending on the preparations and
conditions of study. Cutler and Lloyd (59) reported enhanced
antibody responses to *Candida*-unrelated antigens induced by
cell wall extracts. Similar results were obtained by Domer
et al (67). Cell wall mannans and β-glucan fractions have

also been reported to stimulate antitumor activity of natural killer cells and macrophages (176,215,294,295). The mannoprotein extracts were also capable of inducing lymphoproliferation and production of interferon-γ (IFN-γ). The extracts also generate cell-mediated cytotoxicity in peripheral blood mononuclear cells (PBMC) in human (8,9,339). The physiological relevance as well as the cell components for this non-specific activation of the immune system is unknown. Interestingly, a monoclonal antibody against an oligosaccharide epitope of the immunomodulatory mannoprotein complex reveals a difference in the expression of the complex between yeast- and mycelial-forms of *C. albicans* (338). The epitope was expressed on the surface of yeast cells, but was found on the inner wall layers of the mycelial form rather than on the cell wall surface. These authors suggested that the diminished expression of the epitope on the hyphal surface may be relevant to the proposed increased pathogenicity of the hyphae.

In contrast to the above findings, inactivated *C. albicans* or cell wall extracts of *C. albicans* have been shown to reduce immune responses. Lymphocytes from patients with chronic mucocutaneous candidiasis (CMC) often fail to proliferate in response to mitogens or *Candida* antigens (163,164,349). Whether the immunological abnormality is etiologically important or is secondary to the infection is not clear (348). However, induction of suppression of

antibody formation to T-dependent antigens by formalin-killed
C. albicans has been reported by Rogers and workers (51,52).
The suppressor cells, which appear to be B lymphocytes, can
be elicited by killed *C. albicans* both *in vitro* and *in vivo*.
DTT-extracted cell wall materials from *C. albicans* have also
been shown to non-specifically induce suppressor T cells
which inhibit antibody responses (53). The relationship
between the T-suppressor cell-inducing activity and the B-
suppressor cell activity is not understood. In addition, the
chemical composition of the active moiety in the DTT extract
was not reported. Polysaccharides purified from *C. albicans*
have been reported to induce the production of non-specific
inhibitory factors from human T lymphocytes which block
antigen-stimulated proliferation and development of NK cells
(201). The inhibitory factor blocks the production of IL-2,
the expression of IL-2 receptor, and the synthesis of
interferon by peripheral blood mononuclear cells (PBMC)
(200). Durandy *et al* (74) have reported T cell suppression
induced by *Candida* mannan. These authors showed that
monocytes were required for suppressor activation and that
both CD8$^+$ and CD8$^-$ T lymphocytes were involved.

The presence of inhibitory factors in CMC patient sera
has also been documented by others (88,250,263). At least
part of the inhibitory effect has been attributed to the cell
wall mannan (250,263). In the report by Fischer *et al* (88),
the circulating inhibitor was thermal stable,

nonprecipitatable with ammonium sulfate and could be adsorbed from serum by anti-*Candida* antibody and concanavalin A. These authors suggested that these polysaccharide antigens from *C. albicans* cell wall mannans were involved in specific suppression of cellular functions in chronic candidiasis.

The diverse activities of mannan in its immunoregulatory functions has raised much attention, and several possibilities have been proposed. Cassone (40) suggested that *Candida*-induced immunopotentiation and suppression may be simultaneously expressed but were picked up by various approaches used by different investigators. It is also possible that some of the mannoprotein fractions were crude mixtures of different constituents (including β-glucans) that may obscure the results. Domer *et al* (68) were able to separate mannoproteins on the basis of molecular size and charge, some (14% of total mannan) of which enhanced and others (more than 50% of total mannan) suppressed the *in vivo* antibody response to T-dependent and T-independent antigens. The unseparated mannan had both stimulatory and suppressive effects depending on the dose.

It is thus likely that the different effects of cell wall mannoproteins on the host immune system reside in the fine chemical structures of the preparations. In this context, it should be noted that the methods for preparation of mannan from *C. albicans* by these investigators involved precipitation with Fehling's reagent, resulting in a

structurally modified mannan contaminated with copper, which
has an immunoinhibitory effect of its own (250). The copper-
complexed mannan is also deficient in O-linked
oligosaccharides (249), and has a reduced protein content
(304). The decrease in O-linked oligosaccharides may
influence the assay result (see below).

A more native mannan prepared by complexation with
cetyltrimethylammonium bromide (Cetavlon) was reported to be
a potent stimulator of lymphoproliferation when cultured with
human PBMC (262,263). Interestingly, oligosaccharides
derived from this mannan preparation by weak alkaline
degradation were potent inhibitors of lymphoproliferation
stimulated by *C. albicans* and other antigens. The
oligosaccharides released by alkaline β-elimination are those
of O-linked mannose chains joined through α-1,2-linkages with
occasional terminal α-1,3-linkages (Figure 1). Oligomannosyl
chains of similar size in cell-free supernatant fluids were
detected when mononuclear leukocytes were incubated with
tritiated mannan purified from *C. albicans* (263). It was
proposed that polysaccharide catabolites of fungal mannan may
contribute to the suppression of cell-mediated immunity in
candidiasis (263). On the other hand, some investigators
have suggested that the protein moiety, instead of
carbohydrate, is responsible for the immunosuppressive
ability in the cell wall extracts (37,40,206).

It is clear that cell wall mannan from *C. albicans* has

the potential to influence host immunity in multiple ways. Elucidation of these distinct effects (immunostimulatory and immunosuppressive) of *C. albicans* mannan on its host and its possible role in pathogenesis require further studies. Part of the resolution would be dependent upon the isolation of homogeneous cell wall fractions in their native forms (66). In addition, chemical compositions (protein vs. carbohydrate) of the biologically-active fractions need to be characterized. Furthermore, it is important to consider the possible variable-expression of immumomodulatory molecules on *C. albicans* that are exposed to the host immune systems during various stages of infection (338).

The variable expression of *C. albicans* surface mannan determinants during cell growth has been documented extensively (26,30,118). It is not known whether this variability is caused by the reorganization of pre-existing materials (55,301,343) or by transportation/secretion of newly synthesized materials from the cytoplasm (134,267). In either case, the biosynthesis of mannan is an important consideration.

Mechanisms for glycosylation and secretion of glycoproteins in yeasts have come from studies on *S. cerevisiae* (24,132,252). Cytoplasmic organelles (rough endoplasmic reticulum RER, Golgi, Berkeley bodies) function in a sequence of events, result in the incorporation of glycoproteins into the plasma membranes. Similar mechanisms

of mannoprotein synthesis are indicated in *C. albicans* (12,14). Attachment of mannose residues to protein occurs via a lipid intermediate (dolichol) in the cytoplasm (181,187). In addition, it was suggested that further mannosylation reactions occur at the plasma membrane level (217). Evidence of this comes from the studies of purified membrane preparations of *C. albicans*. The plasma membrane was shown to contain mannan synthetase which incorporates mannose residues in the absence of dolichol phosphate (188,216,299). Other investigators have observed mannan antigens on the exterior cell wall surface as well as on the plasma membrane. These authors suggested the plasma membrane as a site of mannan synthesis (330). Localization of mannan at the surface of yeast protoplasts also supports this view (135).

Secretion of membrane bound materials to their destined cell wall location may involve a specific route. Observations of membranous structures (lomasomes) between the plasma membrane and the inner cell wall layer have been reported in *C. albicans* (274). These vesicular bodies were proposed to have originated from the plasmalemma and perform a secretory function in the formation of the cell wall of *C. albicans*. Other investigators proposed a parallel network of passages through the cell wall for the transportation of synthesized cell wall materials (267). The plasma membrane has also been reported to be associated with other enzymes