



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  $\beta$ -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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**Ren-Kai Li**

**A thesis submitted in partial fulfillment  
of the requirements for the degree**

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in  
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**APPROVAL**

of a thesis submitted by

Ren-Kai Li

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

12/31/92  
Date

Jim E. Cottle  
Chairperson,  
Graduate Committee

Approved for the Major Department

12/31/92  
Date

Alfredo J. Zavala  
Head, Major Department

Approved for the College of Graduate Studies

1/5/93  
Date

Al Brown  
Graduate Dean

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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  $\beta$ -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/creatinine 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 overcome 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.

#### Morphogenesis.

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. guilliermondii* (162). Segal et al (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  $\beta$ -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  $\beta$ -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  $\beta$ -1,3-glucan (110,180), while the synthesis of  $\beta$ -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  $\beta$ -1,6-linkages (306,307). It has been suggested that a large portion of mannoproteins are covalently linked to  $\beta$ -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  $\beta$ -1,3-glucan by the action of  $\beta$ -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  $\alpha$ -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  $\alpha$ -1,6-linkages to form the main chain with side chains of various length attached via  $\alpha$ -1,2- and  $\alpha$ -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  $\beta$ -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- $\alpha$ -mannanase and Endo- $\beta$ -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  $\alpha$ -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 ( $^1\text{H}$  NMR) analysis of mild acid-hydrolyzed fractions indicate that the phospho-oligomannosyl contains homologous  $\beta$ -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  $\beta$ -elimination and cleaves the O-linked polymannose from the protein moiety (Figure 1) (15,22,240). Enzymes such as Endo-H,  $\alpha$ - or  $\beta$ -D-mannosidase, and exo- $\alpha$ -mannanase are also useful for characterization of polysaccharides from yeast mannans (145,248,331,341). Exo- $\alpha$ -mannanase hydrolyses manno-oligosaccharides with  $\alpha$ -1,2- and  $\alpha$ -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  $\alpha$ -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  $\alpha$ -1,3-linkage in addition to four  $\alpha$ -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  $\beta$ -1,2-linkages in one of the serotype A-specific epitopes (303). The relevance of  $\beta$ -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  $\beta$ -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  $\beta$ -linked phosphomannan (303). However, Shibata et al have reported that the  $\beta$ -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  $\beta$ -linkages are found; one located in the acid-stable, and the other in the acid-labile regions (Figure 1) (304). The  $\beta$ -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  $\alpha$ -1,2-linkages in addition to one, two, or three  $\beta$ -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  $\beta$ -1,2- and  $\alpha$ -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  $\beta$ -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- $\gamma$  (IFN- $\gamma$ ). 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<sup>+</sup> and CD8<sup>-</sup> 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,

nonprecipitable 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 immunopotential 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  $\beta$ -glucans) that may obscure the results. Damer 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  $\beta$ -elimination are those of O-linked mannose chains joined through  $\alpha$ -1,2-linkages with occasional terminal  $\alpha$ -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 immunomodulatory 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

such as chitin synthetase (25,73,219), glucan synthetase (288), and  $\text{Na}^+/\text{K}^+$ -ATPase (216). The presence of these enzymes suggests a major role of the plasma membrane in the biosynthesis and secretion of cell wall polymers.

In this report, a monoclonal antibody was developed which specifically recognizes an epitope on the cell surface and plasma membrane of *C. albicans*. The monoclonal antibody was used to determine the expression of antigen on blastoconidia and germ tubes. Antigen expression on other *Candida* species and other fungal species was also surveyed. The antigen distribution was observed at the ultrastructural level in cells fixed by the disparate techniques of traditional chemical methods and freeze-substitution. In addition, antigens were extracted from the cell surface and plasma membranes for chemical characterization. The epitope which specifically reacts with the monoclonal antibody was isolated from the surface of *C. albicans*, purified, and chemically analyzed. The role of this purified antigen and epitope in the pathogenesis of disseminated candidiasis was investigated and discussed.

## Chapter 2

### A CELL SURFACE/PLASMA MEMBRANE ANTIGEN OF C. ALBICANS

#### Introduction

The cell wall and plasma membrane of *Candida albicans* constitute the barrier between its cytoplasm and the external environment. These layers are intimately involved in the shape of the cell, antigenicity and secretion of hydrolytic enzymes. The cell wall of *C. albicans* has thus been a major area of investigation of candidal-host interactions mainly because of the role in adherence to its host (35,195,213), and interactions with host immune systems (36,182). The plasma membrane, on the other hand, is of particular importance in homeostatic control and susceptibility to antifungal agents (301).

The mechanism of transportation and secretion of *C. albicans* cell wall precursors from the cytoplasm to their destined location is unclear (4,85,217,274). Although the biosynthesis of the cell wall of *C. albicans* is not fully understood, information obtained from protoplast regeneration, electron microscopy and chemical determinations have revealed the chemical composition of the cell wall.

These observations have confirmed the importance of the plasma membrane in the synthesis of cell wall layers (63,73,85,178,217,330,343).

Further understanding of the role of the plasma membrane in cell wall biosynthesis requires detailed studies of critical cell wall epitopes which should be associated with the plasma membrane during various stages of cell wall synthesis. That is, in spite of profound chemical, structural and functional differences between the cell membrane and cell wall of *C. albicans*, it has been suggested that certain epitopes may in fact be shared at various times in the cell cycle (134,257).

In this chapter, we determined the presence of an epitope found exclusively on the plasma membrane and also on the cell wall surface of *C. albicans* blastoconidia and germination tubes. The antigen distribution was observed at the ultrastructural level in cells fixed by the disparate techniques of traditional chemical methods and freeze-substitution. In addition, a chemical treatment that removed cell surface antigen without disturbing the membrane was established for differential purification of the antigen from either location.

### Materials and Methods

#### Organisms and Culture Conditions.

*Candida albicans* strain 1 was used in all experiments

unless indicated otherwise. Other clinical isolates of *C. albicans*, *Candida* spp. and other fungi including *Cryptococcus neoformans*, *C. albidus*, *Torulopsis glabrata*, *Rhodotorula* and *Saccharomyces cerevisiae* were from the Montana State University mycology stock collection. Cultures were obtained from frozen stocks and grown on modified Sabouraud dextrose agar (Difco Laboratories, Detroit, MI.) slants. For most experiments, cells were transferred from slants and grown in glucose (2%, w/v)-yeast extract (0.3%, w/v)-peptone (1%, w/v) broth (GYEP) at 37°C under constant aeration by rotation of flasks at 180 rpm (Gyratory incubator, New Brunswick Scientific Co., Edison, N.J.). For immunoelectron microscopy (IEM) of intact yeast cells and preparation of spheroplasts, yeasts were grown in GYEP for 22-24 h at 37°C with aeration, reinoculated into fresh GYEP to a Klett value of 100 (Klett-Summerson photoelectric colorimeter fitted with a No. 66 filter, Klett MFG. CO., INC. N.Y.) and harvested in log phase (Klett value 400). In some experiments, stationary phase cells were harvested (Klett value 550).

Two different media were used for induction of germination. In most experiments, hyphae were induced in a synthetic medium originally described by Lee et al (186) as modified by Sundstrom and Kenny (324) and referred to as modified Lee medium. Cells were grown in the modified medium without bovine serum albumin (BSA) (ImmunoBiologicals, Lisle,

IL) for 48 h at 25°C to obtain yeast-form cells and transferred to prewarmed medium at 37°C containing 1% BSA to promote germination. Cultures were incubated at 37°C and aerated by rotation at 180 rpm. An alternative chemically-defined medium was used in the IEM studies of germinating cells in which yeast-form cells were grown to stationary phase in GYEP at 37°C, inoculated into GM-2 medium (124) at  $2 \times 10^6$  cells/ml and incubated at 37°C under aeration for hyphal induction. Germination was assessed by direct light microscopy and the cells were used if germination exceeded 90% by criteria defined previously (122).

#### Spheroplasts and Subcellular Fractions.

Spheroplasts. Spheroplasts were prepared following procedures described by others (137,361) with slight modifications. Exponential phase yeast cells were washed in 0.1 M ethylenediamine tetraacetic acid (EDTA) (Ph 7.5, disodium salt, Sigma Chemical Co., St. Louis, MO) suspended to 2 ml/g wet weight in 0.1 M EDTA with 0.3 M  $\beta$ -mercaptoethanol (Ph 9.0) and incubated at 24°C for 15 min. Cells were pelleted by centrifugation at  $2,000 \times g$  for 10 min and suspended to the same concentration in 1.0 M sorbitol-0.1 M EDTA (Ph 7.5). Zymolyase 20T (ICN Biomedicals, Inc., Costa Mesa, CA) was added at 80 units/g wet weight of yeast cells and incubated at 37°C for 60 min with frequent agitation. In some preparations for IEM, the  $\beta$ -mercaptoethanol treated

cells were incubated with Zymolyase for only 10 min to remove surface layers. The efficiency of spheroplast formation was determined by counting (hemocytometer) intact cells before and after lysis in 1% (w/v) sodium dodecyl sulfate (SDS, Sigma) as described (108). Spheroplasts were washed in 1 M sorbitol-0.1 M EDTA and either fixed for electron microscopy or processed as described below for mitochondrial fractions.

Subcellular Fractions. Subcellular fractions were prepared as described by others for isolation of mitochondria (361). Spheroplasts, induced by treatment of yeast cells with Zymolyase for 60 min as above, were washed and suspended in 0.25 M sucrose (Sigma), 1.0 mM EDTA and 5 mM [Tris(hydroxymethyl)aminomethane hydrochloride] (Tris-HCl, Sigma) pH 7.5, and homogenized in a Dounce tissue homogenizer at 4°C. After removal of cell debris and intact cells at 3000 x g centrifugation for 10 min, the supernatant material was centrifuged at 15,000 x g to pellet the mitochondrial fraction. Fractions enriched for mitochondria were obtained from continuous sucrose gradients (25-50 % w/v) run at 41,000 x g for 90 min (361) (SW 41Ti rotor, Beckman). The same fractions were used for immunization of animals. This fraction contained mitochondria and other membranous vesicles as determined by electron microscopy.

Plasma Membranes. Plasma membrane fractions were obtained from yeast-form cells as described by others (91).

Stationary phase yeast cells were washed and suspended in an osmotic stabilizer (0.4 M KCl, 20 mM triethanolamine, Sigma, pH 7.0). Cells were broken in a heavy walled centrifuge tube (Corex tubes, Corning Glass Works, Corning, N.Y.) by mixing with 0.45-0.5 mm glass beads on a vortex mixer (123). The suspension was spun at 2,000 x g for 5 min to remove cell debris and the supernatant material was centrifuged at 5,000 x g for 10 min to pellet membranes. The crude membrane pellet was washed three times in the osmotic stabilizer, fractionated by sucrose density gradients (10-60 %) at 23,500 x g for 70 min and resultant bands were collected.

#### Non-specific Trapping of Materials in Membrane Vesicles.

Equal volumes of spheroplast and colloidal gold particles in suspension (i.e., secondary antibody-gold conjugate as described above) which was 10 times diluted in PBS were mixed in a microfuge tube. After 30 min incubation at room temperature, spheroplasts were washed and prefixed in 0.25% formaldehyde (J. T. Baker Chemical Co. Phillipsburg, N.J.). Samples were then fixed and post-fixed in glutaraldehyde and osmium tetroxide, dehydrated and embedded for thin sectioning. Sections were not reacted with the primary Mab, but were contrast-stained and examined by transmission electron microscopy.

Hybridoma and Monoclonal Antibody (Mab).

Immunization. Observations of others in this laboratory showed that mitochondrial-rich fractions induced mice to produce antibodies which reacted with the mitochondrial fraction and with the cell-surface of *C. albicans*. Thus, fractions enriched for mitochondria and other membranous vesicles, and hence referred to as the membranous fraction, were used for 6 weekly intravenous (i.v.) 0.1 ml injections in BALB/cByJ mice. Lowry protein concentration was 4.6 mg/ml. Three days after the last booster injection, mouse sera were checked for agglutinins against intact *C. albicans* blastoconidia, and splenocytes from agglutinin-positive animals were prepared for cell fusion with a mouse non-secreting myeloma cell line (P3X63). Detection of antibody produced by hybrids, cloning by limiting dilution was done as previously described (26). The Mab isotype was determined by Ouchterlony double diffusion test.

Ascitic Fluid Production. To obtain Mab at high concentration for subsequent purification attempts, ascitic fluid containing Mab was induced in BALB/Cbyj mice. Monoclonal cell lines were grown in RPMI 1640 (Gibco Laboratories, Santa Clara, CA) supplemented with 10% fetal bovine serum (FBS, Sigma), washed with RPMI, and  $2 \times 10^6$  cells were inoculated intraperitoneally (ip) into mice which had been injected ip with 0.5 ml of Pristane (2,6,10,14

tetramethylpentadecane, Sigma) 10-15 days earlier. Ascitic fluid which accumulated within 3-5 wk was aspirated from the peritoneal cavity, centrifuged at 1,000 x g and supernatant fluid was stored at -20° C. The concentration of Mab in ascites was titered by the ELISA test.

Precipitin Test. Ouchterlony double diffusion tests were carried out in 1% agarose (Sigma) prepared in 0.15 N sodium chloride. For isotype determination, 10x concentrated hybridoma cell supernatant fluid was put in the center well, and goat antibodies (Sigma) specific for mouse light or heavy chain immunoglobulin determinants were used in the surrounding wells. For antigen identity determination, crude antigen preparation (described below) and ascitic fluid containing different Mabs were used.

Enzyme-Linked Immunosorbent Assay (ELISA) and Agglutination.

Qualitative agglutination tests for the detection of antibodies were done on heat-killed yeast cells as described before (26).

ELISA. ELISA tests (from either Bethesda Research Laboratories, Santa Clara, CA, or Boehringer Mannheim Biochemicals, Indianapolis, IN) were used for detection and titering of Mab in culture fluid and ascites. 96 well microtiter plates (Corning, Corning, NY) were coated with 150  $\mu$ l of yeast cell suspension ( $2 \times 10^6$  cells/ml) in modified

Lee medium at 37° C for germination. At 4 h incubation, the wells, now coated with germinating cells, were washed four times with 0.01 M phosphate buffer saline (PBS, pH 7.2) and 100  $\mu$ l of appropriately diluted test fluid or negative control material were added and incubated at room temperature for 1 h. ELISA detection of mouse Mab was determined following the manufacturer's instructions. In addition to negative controls as indicated above, fresh RPMI medium was used for detecting inappropriate binding of secondary antibody to the cells. An IgM Mab described previously as C6 was used as a positive control (28,29).

#### Immunocolloidal Gold Electron Microscopy (IEM).

Immunocolloidal gold transmission electron microscopy was used to visualize the antigen expression on intact cells, spheroplasts and membranous fractions. Two procedures for fixation of samples were adopted for IEM. Samples were either chemically fixed (30) or freeze-fixed and substituted with anhydrous acetone as described by others (128-130).

Chemical Fixation. In the first procedure, samples were fixed for 30 min in fixative solution: glutaraldehyde (0.5%), acrolein (1%, Sigma), sucrose (0.2%, Sigma) in 0.075 M PBS. After washed twice in deionized water, samples were post-fixed in 1% osmium tetroxide (Ted Pella, Inc. Redding, CA) for 1 h. Samples were then washed in water at 4°C and dehydrated through a series of graded ethanol before

embedding.

Freeze-Substitution. In this procedure, a thin layer of samples was rapidly frozen at  $-192^{\circ}\text{C}$  in a mixture of liquid propane and ethane, the frozen samples were transferred to anhydrous acetone substitution fluid containing 2% osmium tetroxide and 0.05% uranyl acetate at  $-80^{\circ}\text{C}$ . Samples were dehydrated and fixed at  $-80^{\circ}\text{C}$  for 2-3 days and gradually brought up to room temperature ( $22-24^{\circ}\text{C}$ ) (2 h at  $-20^{\circ}\text{C}$ , 2 h at  $4^{\circ}\text{C}$  and then room temperature). This freeze-substitution method was used in fixing yeast cells and spheroplasts. Samples were washed 3 times in anhydrous acetone and embedded in Spurr's resin for thin sectioning (315). Thin sections on nickel grids were reacted with 10 times concentrated culture fluid from a cloned hybridoma cell line, blocked with 3% BSA, reacted with goat anti-mouse secondary antibody conjugated with colloidal gold (10 nm) (Janssen Biotech., Piscataway, N.J.), and finally contrast-stained with lead citrate and uranyl acetate.

Immunofluorescence Assay (IFA).

The assay was modified from that described by Raff et al (272). Mycelium-form cells induced in modified Lee medium were harvested at different incubation times and fixed in 3% (v/v) formaldehyde at  $4^{\circ}\text{C}$  for 12 h. Cells were washed and suspended in PBS, cytocentrifuged onto a glass slide and treated for 5 min at  $4^{\circ}\text{C}$  in 5% acetic acid in 95% ethanol.

Slides were washed in PBS and air dried before adding antibody. Undiluted ascitic fluid containing the Mab or 10 times concentrated culture fluid from the chosen hybridoma clone was added to the fixed cells on the slides and incubated in a moist chamber for 15 min at 37°C. Slides were washed by dipping in PBS several times at room temperature and air dried before adding fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Cappel, Organon Teknika Corp., West Chester, PA) (diluted as manufacturer's suggestion). After incubation for 15 min at room temperature and washing in PBS, cells were examined with a fluorescence microscope (Nikon Episcopic-fluorescence microscope, type 104, equipped with a high pressure mercury lamp 100W/2).. The same procedures were used for yeast and hyphal forms. Samples treated with normal mouse serum instead of primary antibody were incubated under the same conditions as a control for non-specific binding of secondary antibody.

#### Crude Antigen Extraction.

The agglutinin-specific antigen was solubilized from yeast cells by hot phenol extraction (358) as done previously on *C. albicans* (57). Briefly, log phase yeast cells were suspended in a 1:1 (v/v) mixture of phenol and water prewarmed to 60°C and shake vigorously at 60°C for 30 min. After cooled to 10 °C, the suspension was centrifuged and the

aqueous phase was collected, dialyzed against deionized water, lyophilized and referred to as the phenol extract.

#### Partial Antigen Characterization.

For characterization of the extracted antigen, the phenol extract was dissolved in PBS at a concentration of 5 mg/ml and subjected to boiling at 96°C for 10 min. For enzymatic treatment, antigen was solubilized at 5 mg/ml in 0.05 M Tris-HCl buffer Ph 7.8 with 5 Mm CaCl<sub>2</sub> and the following enzymes were added: Pronase B (Calbiochem-Behring Corp, La Jolla, CA) 9 units/ml, trypsin (Sigma, from bovine pancreas, Type III) 2.2 units/ml, and proteinase K (Sigma, Type XI) 2.3 units/ml. A standard substrate, azocoll (Sigma), was used at the same concentration (5 mg/ml) as a positive control for each enzyme activity (46). After incubation at 37°C for 2 h with agitation, solutions were either tested directly for antigen by reactivity against the Mab in ascitic fluid in an Ouchterlony double diffusion test or boiled prior to testing. Enzymes and heat-treated enzymes alone in the same buffer were used as controls for possible cross-reactions with the Mab in the double diffusion test.

Intact yeast cells were also treated with heat or proteolytic enzymes as above and examined for surface antigen activities by agglutination and IFA. *C. albicans* strain 1 grown in GYEP for 24 h was washed and suspended in the same buffer described above at a concentration of  $2 \times 10^6$  cells/ml

and treated with various enzymes at 37°C for 2 h. Digestion by enzymes were terminated by boiling for 10 min. Cells were washed in PBS and tested for antigen presence by IFA and agglutination. Untreated and heat treated cells were used as controls.

#### Tunicamycin Treatment.

Tunicamycin (Sigma) dissolved in dimethylsulfoxide (DMSO) was added (final concentration 10 µg/ml) to a yeast culture at different growth phases. At different time intervals after the addition of tunicamycin, surface expression of antigen was tested by agglutination. Freeze-substitution electron microscopy was also used for detection of non-surface antigen.

#### Immunoelectrophoresis.

Immunoelectrophoresis of the crude antigen was carried out on 1 % (w/v) low electroendosmosis agarose (Sigma) gel in different buffers at various pH values (25 mM citrate buffer, Ph 5.0; 10 mM PBS, Ph 7.2; Tris-barbital buffer, Ph 8.8, Gelman Sciences Inc.; and 50 mM Tris-base pH 10.0). Phenol extract in each buffer at 10 mg/ml was applied to a well and electrophoresed for 3 h at DC 4V/c. Troughs were cut into the agarose and monoclonal antibody was added and allowed to diffuse and react with the antigen in a moist chamber at 24°C for 72 h.

## Results

### Mab Characterization.

Four stable hybridoma cell lines were obtained which reacted strongly against *C. albicans* strain 1 as determined by ELISA. One which was chosen for further investigation was cloned by limiting dilution, recloned and designated Mab 10G. The ascites induced in BALB/cByJ mice by the cloned hybrid gave an ELISA titer of 2,400. The Mab 10G belongs to the IgM class with kappa light chains as determined by Ouchterlony double diffusion with specific antisera. No identity was observed between antigens recognized by Mab 10G, C6 and H9 (Figure 2).

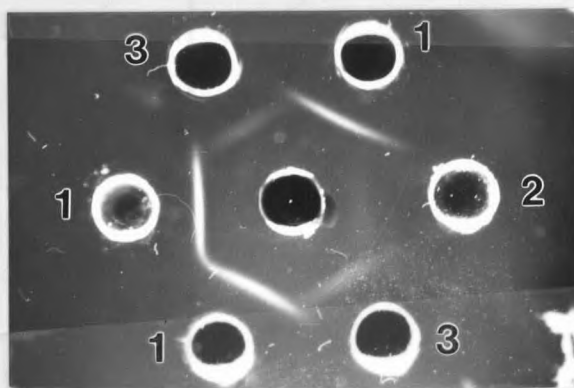


**Figure 2.** Ouchterlony double diffusion test. Reactions of nonidentity were observed in precipitant bands that formed in between crude antigen preparation (phenol extract, center well) with Mabs C6 (1), H9 (2), and 10G (3).

Expression of 10G Antigen of *C. albicans*.

Yeast Form Cells. Surface expression of the epitope recognized by Mab 10G (i.e., Ag10G) was detected by agglutination and IFA of whole cells of *C. albicans* strain 1. Antigen distribution at the ultrastructural level was further investigated by IEM in cells fixed by two different fixation procedures. Similar results were obtained from cells fixed by either glutaraldehyde or freeze-substitution. Ag10G was usually found randomly distributed on the plasma membrane but had a patchy distribution on the wall surface of log phase yeast (Figure 3). The ultrastructural preservation of plasma membranes, nuclei, wall and most cytoplasmic contents were improved by freeze-substitution techniques compared to traditional chemical fixation. To obtain further information on strain and species specificity of expression of the antigen, other isolates of *C. albicans* as well as other *Candida* spp. and other fungal species were examined for surface expression of the antigen by the agglutination test. The following organisms gave positive agglutination reactions: all 15 *C. albicans* clinical isolates and one isolate each of *C. stellatoidea*, *C. tropicalis*, *C. lusitaniae* and *C. intermedia*. No antigen was detectable by agglutination of other fungi which included *Candida utilis*, *C. krusei*, *Cryptococcus neoformans*, *C. albidus*, *Torulopsis glabrata*, *Rhodotorula* spp. and *Saccharomyces cerevisiae*. One

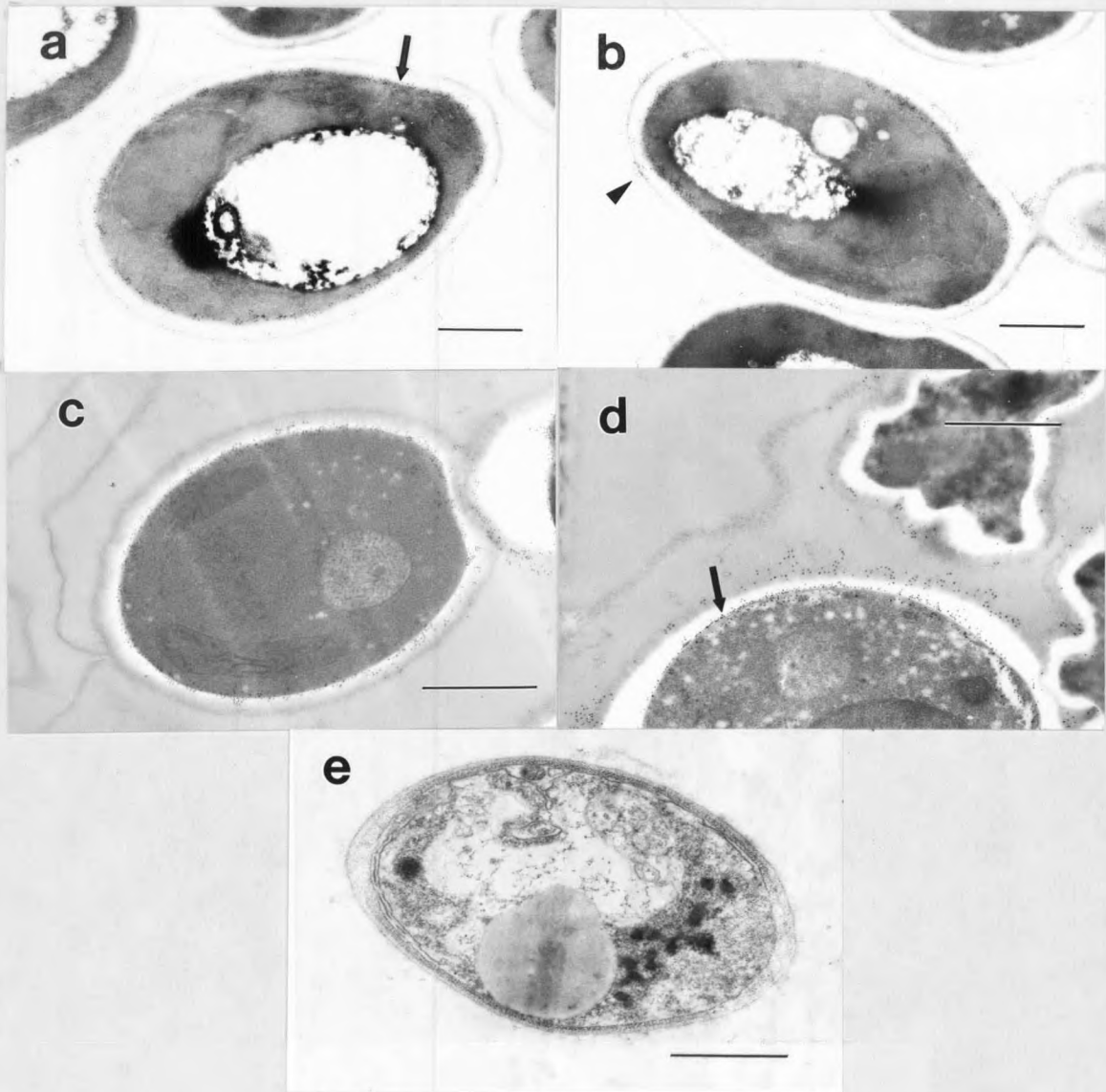


**Figure 3.** Distribution of 10G epitope in exponential-phase *C. albicans* yeast cells fixed chemically as compared to freeze-substituted cells. (a,b) Cells were fixed with glutaraldehyde and osmium tetroxide. Antigen was equally distributed near or on the plasma membrane (arrow) but unevenly on the the cell wall surface (arrowhead). (c,d) Cells fixed by freeze-substitution show similar patterns of antigen distribution and better detail of membrane association of the antigen (arrow). (e) Cells stained with culture fluid from a non-secreting cell line instead of the 10G hybridoma cell line. Bars, 1  $\mu$ m.

of the agglutination-positive isolates of *C. albicans* and one strain of *C. lusitaniae* were examined by IEM and a similar pattern of antigen distribution was observed (Figure 4).

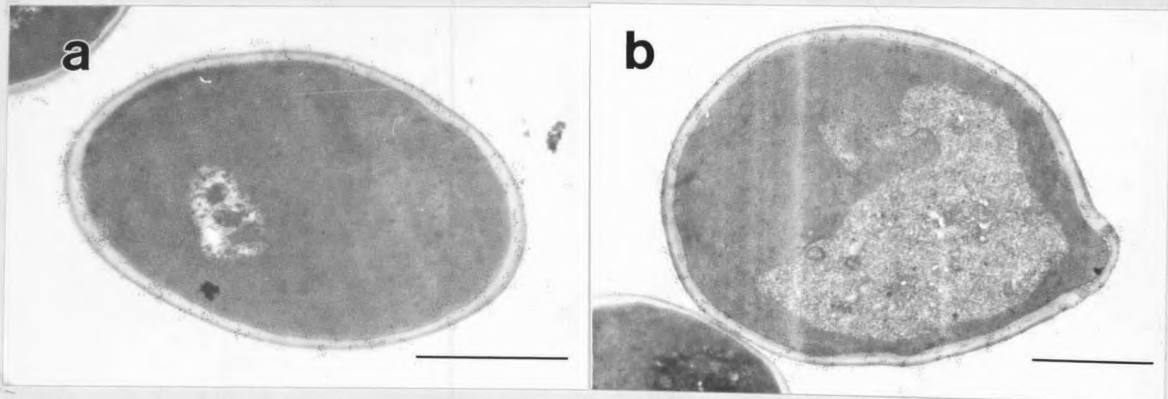


**Figure 4.** Expression of 10G antigen on other *C. albicans* strain (left) and *C. lusitaniae* (right). Similar pattern of antigen distribution is observed as compared to *C. albicans* strain 1 (Figure 3). Bars, 1  $\mu$ m.

Tunicamycin-Treated Yeast Cells. Tunicamycin inhibited the surface expression of antigen, as demonstrated by the agglutination assay, if added to cultures at early log phase. No inhibition was observed if added after 4 h incubation of cultures. TEM of agglutination-negative yeasts revealed a low level of membrane antigen as compared to untreated cells. The antigen was absent from cell walls of some yeasts and present in cell wall layers in others. In some treated cells, accumulation of antigen-containing vesicles was observed. Other signs of cell abnormalities were also noticed (Figure 5).

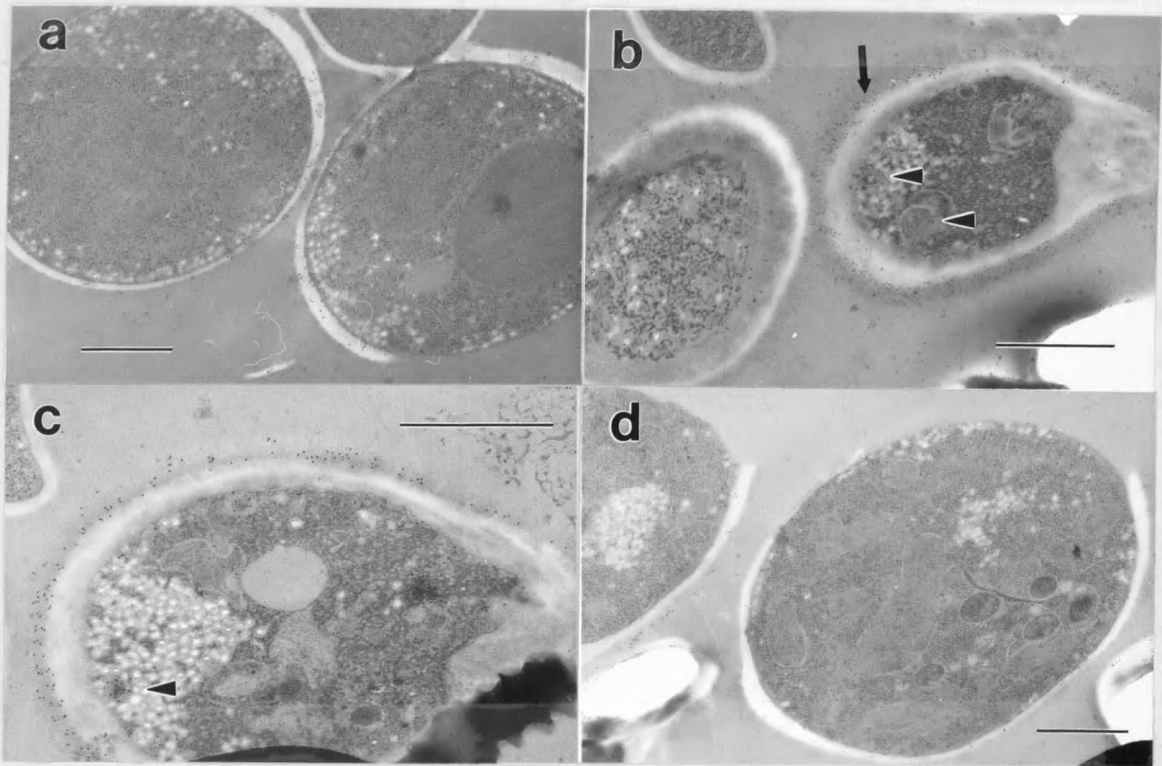


**Figure 5.** Antigen expression on tunicamycin-treated yeast cells. (a) Control group (normal cells) without tunicamycin treatment. Note the abundance of colloidal gold particles, peripheral distribution of glycogen granules, and ribosomes on rough endoplasmic reticulum (RER). (b, c, d) Cells treated with tunicamycin. In some cells, antigen is observed in the outer layer of the cell wall below the fluffy outermost layer (b, arrow). Antigen is absent from the plasma membrane in most cells. Accumulation of antigen-containing vesicles is shown (b, c, arrow heads). Also note the abnormal accumulation of vacuoles, glycogen granules, and loose ribosomes from RER (d). In some cells, loose and expanded cell walls with membranous figures in the cytoplasm were observed (b). Bars, 1  $\mu$ m.

Germinating Cells. Surface expression of antigen on hyphae of *C. albicans* was examined by IFA. Immunofluorescence of germinating cells was typically dense on the mother yeast cells throughout the incubation time. Antigen expression on the hyphal surface was not detectable

for the first 2 h of incubation but became evident as germination proceeded (Figure 6).

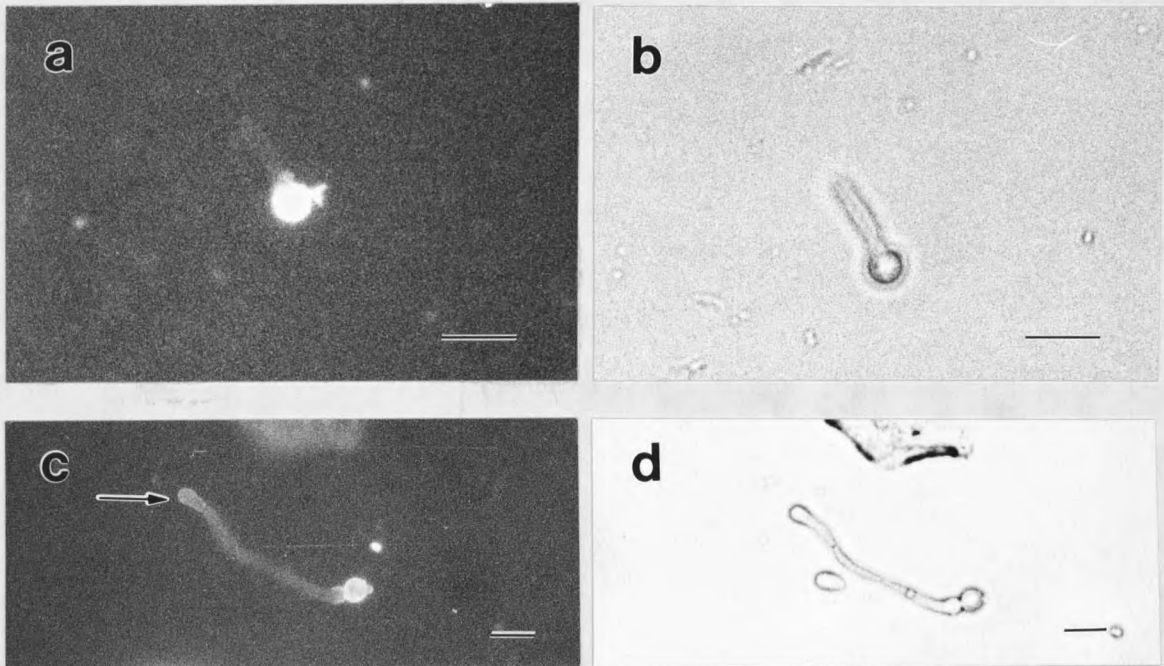


**Figure 6.** Immunofluorescence staining of 10G antigen during germ tube development. (a) At 2 h germination in modified Lee medium, no antigen was detected on germ tubes; (b) bright field image. (c) At 5 h development, antigen became evident along the entire surface of germ tubes and especially on the mother cell, the initial portion of the germ tubes, and the growing apical region (arrow); (d) bright-field image. Bars, 10  $\mu$ m.

These observations were subsequently confirmed by IEM. Stationary phase *C. albicans* cells grown in GYEP were transferred to GM-2 medium and allowed to germinate for up to 8 h. At various incubation times, germinating cells were fixed with glutaraldehyde and prepared for IEM as described above. At time 0, stationary phase cells had increased surface expression over log phase cells (Figure 7a as

compared to Figure 3). Throughout the entire incubation time allowed for hyphal development, antigen expression on parent cells was more dense on the cell wall surface than on the plasma membrane. The development of antigen on germ tubes was first observed by 2 h incubation. Unlike parent cells, the antigen expressed on germ tubes was primarily in the subsurface area near or on the plasma membrane (Figure 7b). At 6 h, antigen became obvious on the cell surface and remained dense in the subsurface area (Figure 7c). Control experiments in which cells were treated with culture fluid of the non-secreting cell line did not show colloidal gold above background level (Figure 7d) ( $<10$  particles cell<sup>-1</sup>).

Spheroplasts. Log phase yeast cells were treated with  $\beta$ -mercaptoethanol and subjected to Zymolyase digestion for either 10 or 60 min. The efficiency of spheroplast formation after Zymolyase digestion for 60 min was consistently over 90%. Samples were freeze-substituted with acetone and prepared for IEM. In cells treated with  $\beta$ -mercaptoethanol and Zymolyase for 10 min, antigen was removed from the surface but remained undisturbed on the plasma membrane. The fibrillar outer cell wall layer was also removed by this treatment (Figure 8a). Presence of organelles which were released from lysed cells were occasionally observed. After 60 min incubation in Zymolyase, no cell wall was observed, yet antigen was still associated with cell membrane (Figure

8b). The ultrastructural preservation of spheroplast organelles was generally improved compared with whole yeast cells.

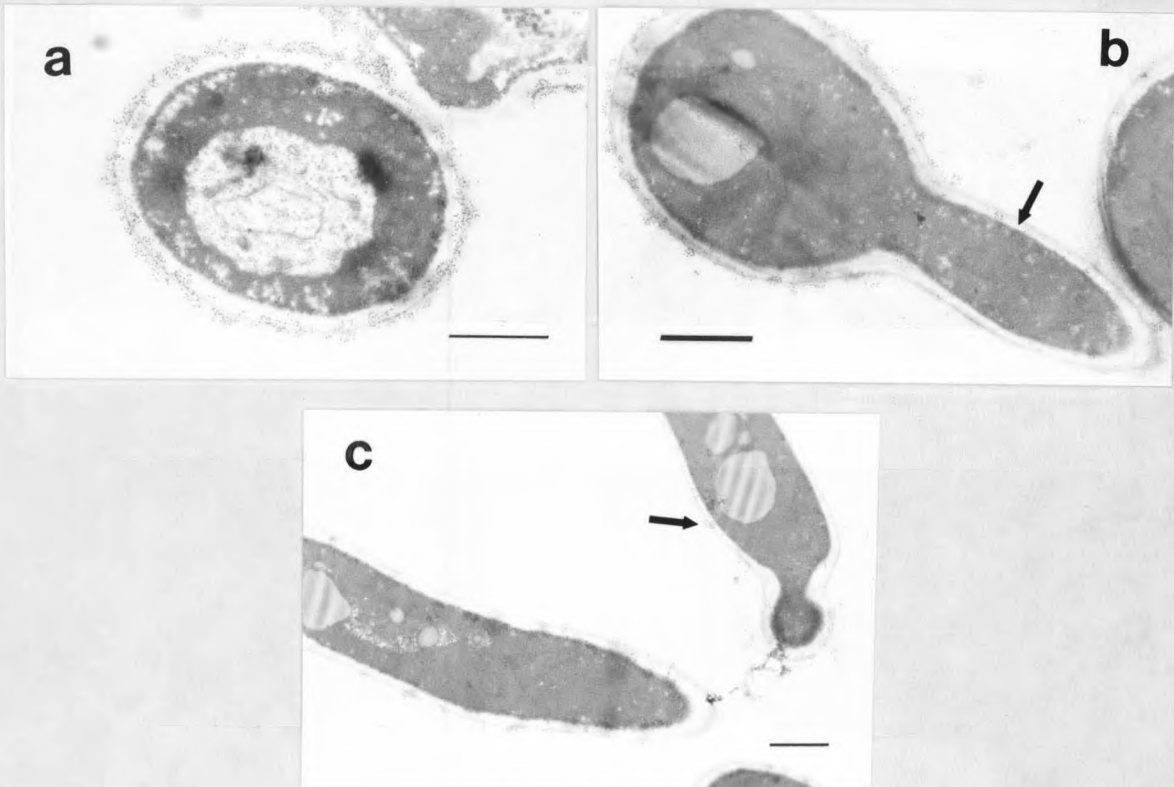


**Figure 7.** Distribution of 10G antigen during germ tube development as determined by IEM. Stationary-phase yeast cells were placed into germination conditions, harvested at various time intervals and fixed with glutaraldehyde and osmium tetroxide. (a) At time 0, epitope expression was especially heavy on the cell wall surface. (b) After 2 h germination, antigen remained densely distributed on the mother cell wall surface, whereas on berm tubes it was primarily expressed into the subsurface area near the cell membrane (arrow). (c) At 6 h, antigen became evident on the germ tube surface (arrow). Bars, 1  $\mu$ m.

**Plasma Membranes.** To further confirm the association of the antigen with the plasma membrane, lysed spheroplasts and

plasma membrane fractions were examined for the presence of antigen. Lysis of spheroplasts was induced by suspension in 0.5% glutaraldehyde. In glutaraldehyde fixed samples of spheroplasts, antigen was mostly found in vesicle structures but not on fragmented membranes (Figure 9a). The vesicles apparently formed spontaneously during glutaraldehyde lysis of spheroplasts. The antigen was also not associated with the plasma membrane in membrane fractions (Figure 9b). When lysed spheroplasts were freeze-fixed and substituted in acetone, antigen was observed evenly distributed throughout the membranes (Figure 9c).

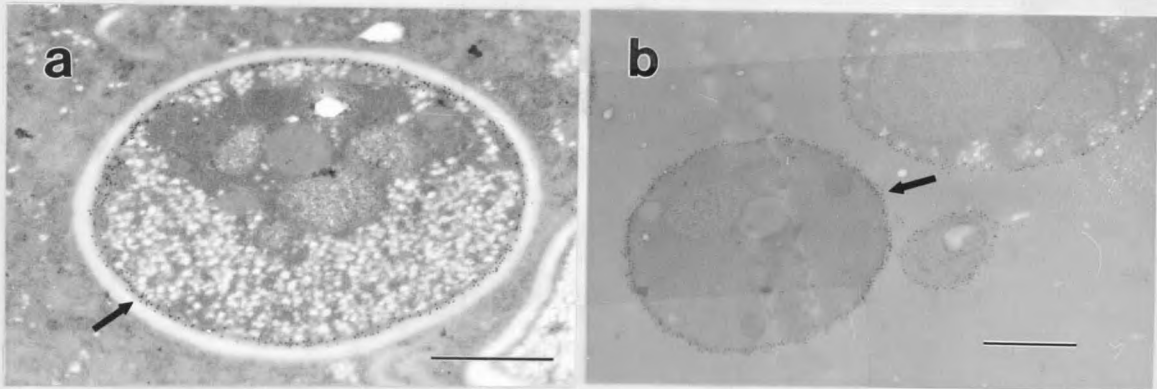


**Figure 8.** Effect of Zymolyase treatment on the 10G antigen. Yeast cells were treated with Zymolyase for either 10 min (a) or 60 min (b), then samples were freeze-substituted and examined by IEM. The antigen was removed from the cell wall surface, while antigen on the plasma membrane was unaffected (arrows). Bars, 1  $\mu$ m.

**Mitochondria.** Mitochondria-enriched fractions were also investigated using immunocolloidal gold electron microscopy in an attempt to explain their immunogenic nature. Fractions

of mitochondria and membranes which co-purified with mitochondria were observed, but antigen was detectable only within single and multilamellar membrane vesicles (Figure 9d).

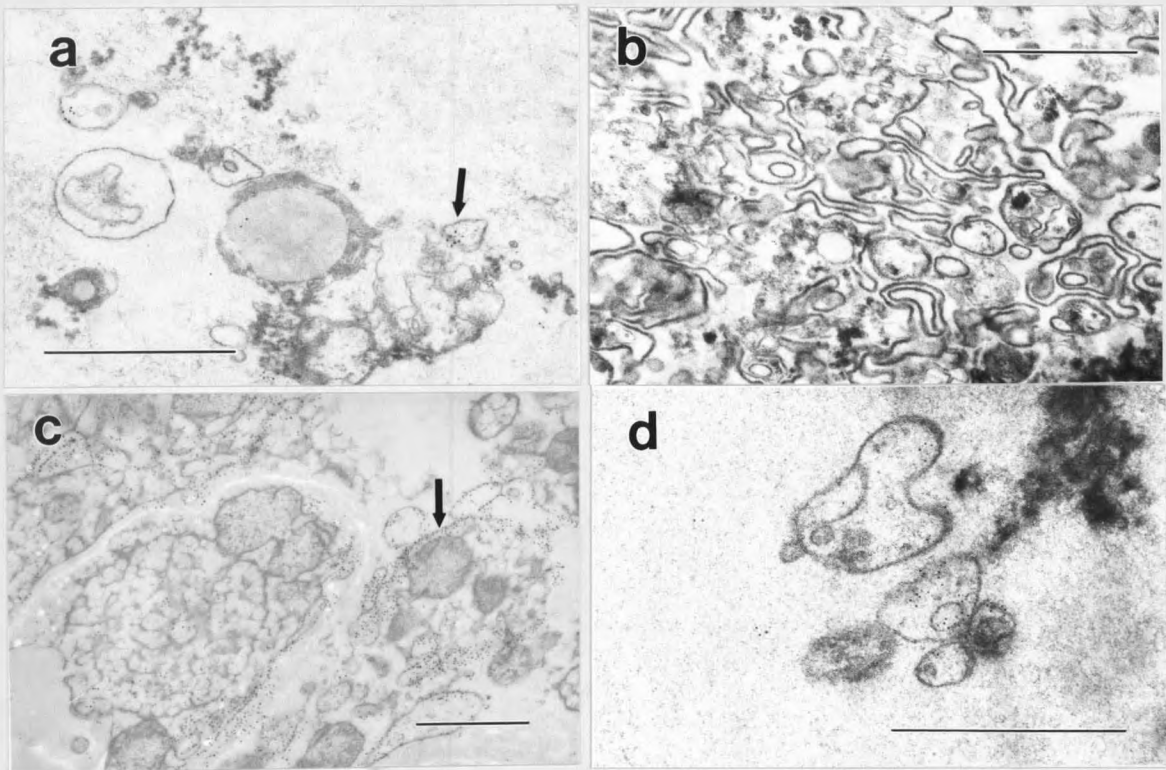


**Figure 9.** Association of antigen 10G with plasma membranes. Lysed spheroplasts (a) and membrane fractions (b) fixed by glutaraldehyde. Antigen was not found on membrane fragments but was associated with vesicles (arrow). (c) Lysed spheroplasts fixed by freeze-substitution showing antigen association with the plasma membranes (arrow). (d) Mitochondria-enriched fractions in which variously sized vesicles co-migrated and trapped the antigen during spontaneous vesicle formation. Bars, 1  $\mu$ m.

#### Non-specific Trapping of Materials in Membrane Vesicles.

Preliminary observations showed that sera from mice

immunized against mitochondrial fractions agglutinated whole cells as well as mitochondrial fractions, it was postulated that the fractions used for immunization may contain cytoplasmic substances other than mitochondria. Under the electron microscope, membrane vesicles which contain antigen were observed in the mitochondrial fractions. The nature of these vesicles were speculated to be from the plasma membrane during lysis of the spheroplasts.

Spheroplasts were incubated with colloidal gold particles without the osmotic balance of sorbitol. In the absence of Mab, extracellular gold particles were sometimes observed in membrane vesicles. Cytoplasmic components were also trapped in plasma membrane vesicles during their formation. This non-specific trapping of materials into the vesicles may be explained by the multilamellar membrane vesicles formation during osmotic lysis of the spheroplasts (Figure 10).

#### Characterization of 10G Antigen.

Proteolytic Enzymes and Heat. The hot water-phenol extract of *C. albicans* was subjected to chemical and physical treatment and subsequently tested for antigenic reactivity against Mab 10G in Ouchterlony double diffusion. Precipitin bands of variously treated phenol extract showed identity with untreated material. The antigen reactivity was not altered by treatment with heat at 96°C for 10 min or

proteolytic enzymes (Pronase B, trypsin, and proteinase K).

Yeast cells grown in GYEP broth were also treated under the same conditions and screened for surface antigen activities by agglutination. Both the treated yeasts and untreated control cells showed the same degree of agglutination.

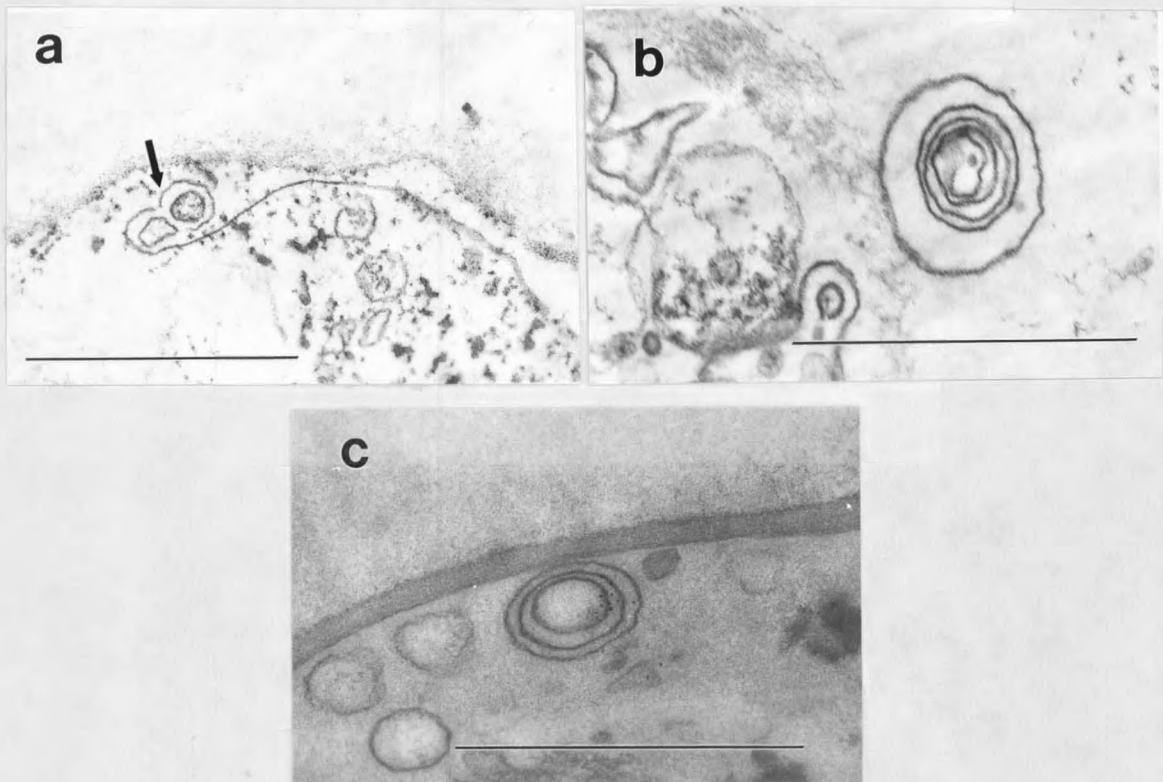


**Figure 10.** Formation of vesicles and non-specific inclusion of extracellular substances. Spheroplasts were incubated with colloidal gold for 1 hour before fixation in the absence of Mab 10G. (a) Cytoplasmic components were trapped in plasma membrane vesicles during their formation (arrow). (b) Multilamellar membrane vesicles sometimes formed during osmotic lysis of the spheroplasts. (c) Extracellular gold particles were non-specifically trapped inside the membrane vesicles. Bars, 1  $\mu$ m.

Electrophoretic Characteristics. Immuno-electrophoresis of crude antigen extract (phenol extract) revealed smear bands of antigen migrating toward the anode in the electrical field in both acid and alkaline environment (Figure 11). At pH 7, the antigen does not migrate in the electric field.

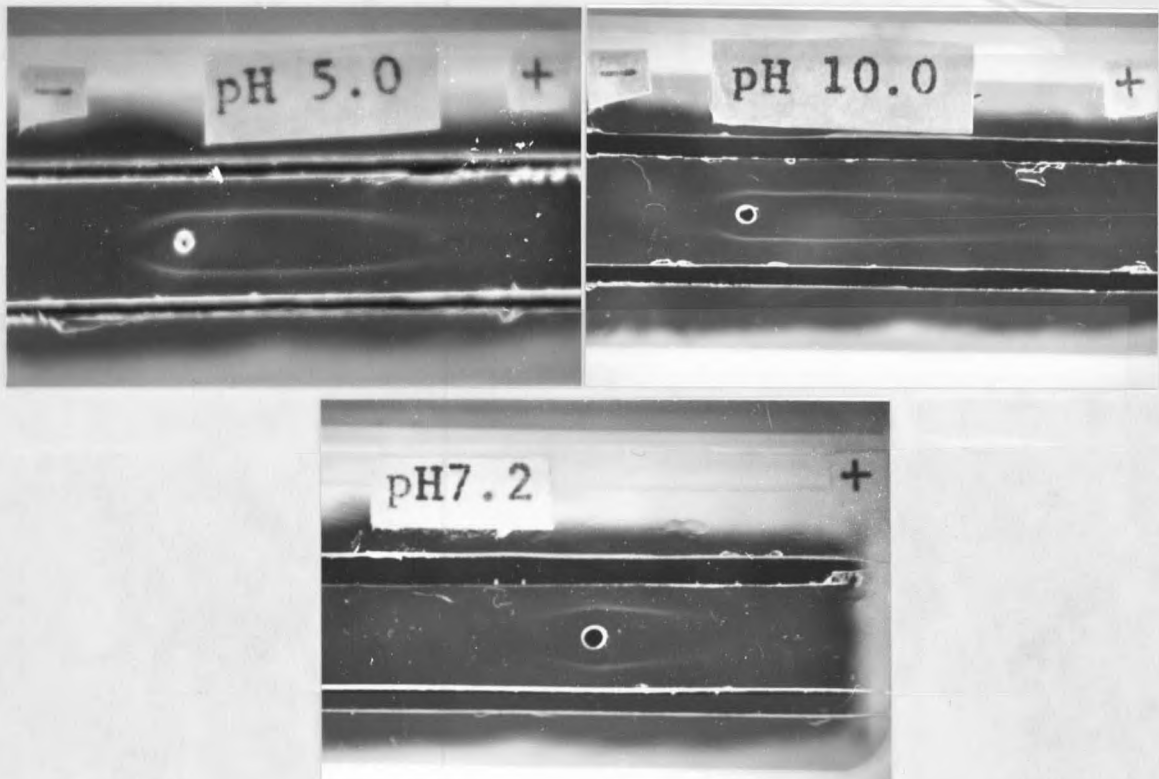


**Figure 11.** Immuno-electrophoresis of the aqueous phase of phenol extract of whole yeast cells. In both acid (pH 5.0) and alkaline (pH 10.0) buffers, the antigen migrates toward the anode.

#### Discussion

Among the many approaches to understanding cell wall structure and development of *C. albicans*, monoclonal

antibodies allow investigators to follow the expression of a particular determinant. In the present study, an IgM monoclonal antibody, with kappa light chains, and designated 10G, was isolated which agglutinates *C. albicans* yeast cells. In addition to the cell surface location of the antigen to which Mab 10G is specific, the appearance of the immunodeterminant also on the plasma membrane was rigorously documented. These findings are not unique to a single strain of *C. albicans* because the same pattern of expression was found on other isolates of *C. albicans*. My results indicate that Ag 10G is a common antigen in the species *C. albicans* and is also expressed in selected other *Candida* species (*C. stellatoidea*, *C. intermedia*, *C. tropicalis*, and *C. lusitaniae*). The antigen is not universal to all *Candida* species because it is not expressed by *C. utilis* and *C. krusei*. In addition, the antigen was not detectable in other yeast genera. However, the absence of reactive epitope on the cell surface of *C. utilis*, *C. krusei* and other fungal genera as demonstrated by agglutination test did not exclude the possibility of intracellular expression.

It has been suggested by investigators, who used only immunofluorescence microscopy, that the same polysaccharide epitope recognized by a monoclonal antibody was located on the cell surface and on the plasma membrane of *C. albicans* (134,257). Another group (330), who used ferritin-labeled antibody, localized a mannan antigen on the exterior cell

wall surface as well as in the cytoplasm near the cytoplasmic membrane. The latter location was regarded as the site of mannan synthesis and it was suggested that the mannan antigens were transported from the cytoplasm via channel-like structures in the cell wall to the final deposits in the exterior cell wall layer. Similar speculation has been reported by others on cellular excretion of glycoproteins through the cell wall of *C. albicans* (267). These workers suggested that antigen emergence at the cell surface correspond to patches of the antigenic material in the fuzzy coat of the cell surface. Although channel-like structures were not observed in our studies, it may be that the patchy distribution of the 10G antigen on the cell wall surface represents the site of antigen transportation. However, chemical analysis of the antigens at both locations is needed to determine whether the plasma membrane antigen is a cell wall precursor or is an unrelated, cross-reactive substance. Treatment of the cells with  $\beta$ -mercaptoethanol and Zymolyase selectively releases the antigen from the cell wall and will allow differential purification of the antigen from both locations for subsequent chemical comparisons.

Information on yeast wall synthesis comes primarily from studies on *Saccharomyces cerevisiae* (252,253,344,345). In *C. albicans* mannosylation reactions might occur at the plasma membrane level (217), and enzymes associated with the cell wall matrix may modify the wall during growth and hyphal

transformation (17,162,189). In our observations, the early appearance of antigen on the membrane of germ tubes and later development on the surface suggests a growth-dependent expression of the antigen during hyphal development. In addition, the dense expression of the antigen corresponded with the growing apical region. We also noted that in yeast-form growth in GYEP, antigen expression was predominantly on the plasma membrane in log phase and primarily on the cell surface of stationary phase cells. Expression of cell surface antigenic determinants may vary as a function of growth phase or morphological states (28,31,310,324,343) and our observations suggest that specific sites of antigen location may be related to structural modifications and/or cellular transportation of a cell wall component during yeast growth and early stages of germination. Ascribing antigen location at the ultrastructural level to specific cellular sites by results obtained from a single fixation procedure is open for criticism. Conventional chemical fixation using glutaraldehyde followed by osmium tetroxide may induce artifactual enlargement of dolipore septa in various basidiomycetes (130). Using freeze-substitution fixation, we obtained essentially identical patterns of antigen distribution as with glutaraldehyde fixation which gives strong support for the cell wall/cell membrane locations of antigen 10G. In addition, freeze-substitution gave apparently better preservation of most of the intracellular

organelles, plasma membranes and the fluffy outer layer of the cell wall of *C. albicans*. One limitation of the method was that only a low percentage of intact yeast cells showed acceptable preservation. The majority of spheroplasts, however, were well preserved. Hence, total or partial loss of cell wall layers may allow faster freezing and better penetration of embedding agents.

Comparison between the two fixation procedures on lysed spheroplasts suggest that although the antigen is associated with the plasma membrane, it is not well anchored. The presence of the antigen on plasma membranes fixed by freeze-substitution but not on membranes fixed by traditional means implies that the antigen becomes dissociated from the membrane during the many washing steps in the glutaraldehyde fixation procedure. A loose association between the antigen and the plasma membrane may explain the absence of antigen on membrane fractions (Figure 9b).

During preparation of mitochondria-enriched fractions, membrane vesicles spontaneously form and can trap antigen released from the cell membrane as demonstrated by electron microscopy. Spontaneous vesicle formation and non-specific trapping of substances by the vesicles has also been observed by others during investigations on *S. cerevisiae* (47,91). In addition to explaining the specific immunogenicity of mitochondria-enriched fractions in our studies, these results indicate that vesicles may be artifactually created during

cellular fractionation procedures. Furthermore, such vesicles may erroneously be thought to be associated with various cytoplasmic substances which were, in fact, passively trapped during vesicle formation.

The antigenic reactivity of either phenol extract or intact yeast cells was not altered after treatment with heat or proteolytic enzymes. These data, together with the behavior of the phenol extracted antigen in immunoelectrophoresis, indicate that antigen 10G is not a protein. Experiments for isolation and purification of the epitope are now in progress which will allow chemical comparisons between the cell surface antigen with that of the plasma membrane.

### Chapter 3

#### CHARACTERIZATION OF A CELL SURFACE EPITOPE ON C. ALBICANS

##### Introduction

The cell wall surface of *Candida albicans* are involved in the initial contact with its hosts and subsequent interactions with host defense mechanisms (76,162,213,293,296,309,314). Mannoproteins constitute a large proportion of the cell wall components (14,89,301) and are proposed to be involved in the pathogenesis during infections (37,263). Many studies which have been emphasized on the cell wall composition have suggested a general structure of *C. albicans* cell wall mannans (14,174,178,235,303). However, current knowledge in mannoprotein synthesis and its role in the pathogenesis of different forms of candidiasis is still incomplete. Because of the complex structure of proposed cell wall mannans, determination of the functional role and the interactions of cell wall mannans with host tissues would largely depend on the study of specific structures.

Structural determinants in the carbohydrate moiety of mannans were suggested to be associated with adherence

ability to host tissues (236). Evidence also suggested that carbohydrate determinants in the cell wall mannans may participate in the immunomodulation of host immune systems (37,66,250,251,262,263). These data strongly indicate a role of mannan polysaccharides at various levels of interaction with host tissues.

In a previous communication (198) (see also Chapter 2), we reported that an antigen recognized by a monoclonal antibody (Mab 10G) was found on the cell surface and also on the plasma membrane of *C. albicans*. The expression of this antigen was mainly on the plasma membrane of exponential phase yeasts, and on the cell wall surface of stationary phase yeasts. To determine the chemical structure of the epitope recognized by Mab 10G, antigens from the cell wall and plasma membranes were sequentially extracted and characterized. The epitope was isolated from the cell wall extract, purified, and its structure was determined. The significance of the epitope and the possible application of the monoclonal antibody is discussed.

### Materials and Methods

#### Organisms and Culture Conditions.

*C. albicans* strain 1 was used throughout these experiments. Medium and culture conditions were those described in Chapter 2.

### Purification of Monoclonal Antibody (Mab).

The identification of the Mab (Mab 10G) and the production of mouse ascites containing Mab 10G used in these studies was described in Chapter 2. Mab in the ascitic fluid was purified by use of an ion-exchange HPLC column (ABx, 15 micron, 10mm x 25 cm, J.T.Baker Research Products, Phillipsburg, NJ.) that was connected to a Beckman model 332 gradient liquid chromatograph equipped with model 110A pumps. The ascitic fluid was diluted three times in 0.25 mM 2N-morpholinoethane sulfonic acid (MES) buffer at pH 5.5 before applying to the HPLC column. Fractions were eluted by a linear gradient of solution A (0.25 mM MES, pH 5.5) to 100% of solution B (1.0 M sodium acetate, pH 7.0) at a flow rate of 1 ml/min. Fractions (1.5 ml/tube) were monitored by absorbance at 280 nm and peaks containing the Mab were confirmed by agglutination of whole yeast cells (Chapter 2). Fractions containing the Mab were pooled, and total protein concentration was determined by BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL) using BSA as a protein standard.

### Crude Antigen Extraction.

Methods for sequential fractionation of antigens from the cell wall and plasma membranes were established following procedures described under the subtitle "Spheroplasts and subcellular fractions" for the isolation of spheroplasts in

the Materials and Methods in Chapter 2. Briefly, exponential phase yeast cells were treated sequentially with  $\beta$ -mercaptoethanol and Zymolyase 20-T in the presence of a mixture of protease inhibitors which included antipain, chymostatin, phosphoramidon, leupeptin, elastatinal and pepstatin A (Sigma) at 2.5  $\mu$ g/ml each and phenylmethylsulfonyl fluoride (PMSF, Sigma) at 1 mM. All of the protease inhibitors were first prepared as stock solution in dimethyl sulfoxide (DMSO, Sigma). The resulting spheroplasts were washed and then extracted with deionized water in the presence of the protease inhibitors. After centrifugation, the pellet was suspended in 0.7% Triton X-100 and shaken vigorously at 37°C for 1 h. The soluble extracts from each treatment ( $\beta$ -mercaptoethanol, Zymolyase, deionized water and Triton X-100) were collected separately and designated as fractions I, II, III and IV, respectively. A scheme of this fractionation procedure is illustrated in Figure 12. Fractions I-IV were clarified by centrifugation at 50,000 x g for 1 h. The supernatants were dialyzed against deionized water and lyophilized.

#### Immuno-affinity Chromatography.

This method was used in an attempt to purified the native form of antigen from crude preparations without chemical treatment. HPLC-purified Mab 10G was conjugated to Sepharose 4B (Sigma) through cyanogen bromide activation

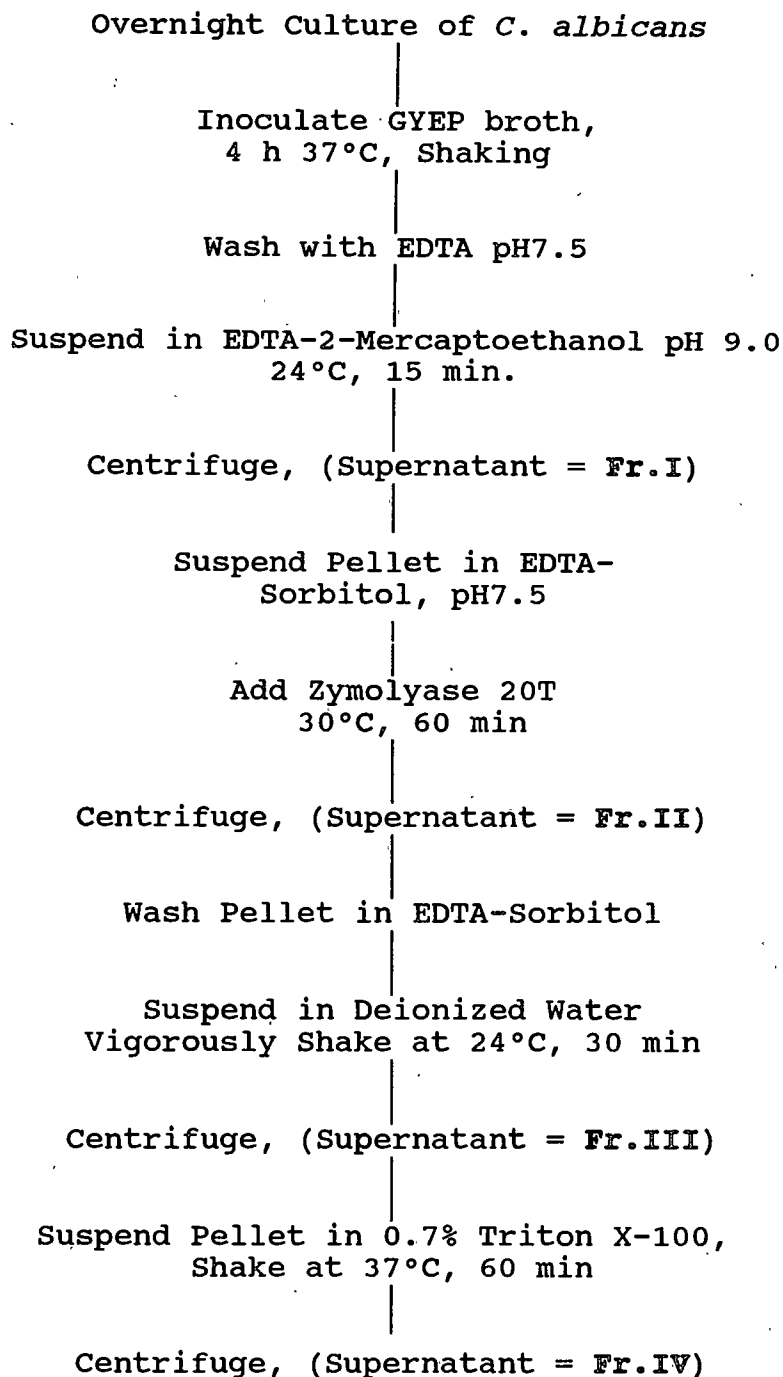


**Figure 12.** Scheme for sequential fractionation of antigens from *C. albicans* cell wall and plasma membranes (see text for details).

according to methods described elsewhere (142,175,266). Briefly, beads were washed in deionized water for two times. Cyanogen bromide (CNBr, Sigma, 0.5 g/5 ml of beads) was added to the suspension and the pH was adjusted and kept at 11 by adding drops of 0.4 N sodium hydroxide constantly. The temperature of the suspension was kept at 20°C by adding ice intermittently. When the pH and temperature became stable, the suspension was cooled to 4°C rapidly by adding ice. Activated beads were washed rapidly with 7 to 10 times volume of borate buffer (0.01 M, pH 9.5, with 0.15 N NaCl) at 4°C. HPLC-purified Mab (2.5 mg protein/ml) was added to the same volume of beads and allowed to react for 12 h at 4°C with constant rotation. Beads were washed three times in PBS before packing (0.8 x 8 cm). Columns with Sepharose beads conjugated with normal mouse serum (NMS) and BSA were used as control groups.

Conjugation of the monoclonal antibody to the Sepharose beads was demonstrated in two ways. First, the ability of HPLC-purified Mab 10G solution to agglutinate heat-killed yeast cells was lost after the conjugation procedures. Second, the attachment of heat-killed yeast cells to Mab-conjugated beads but not to NMS or BSA conjugated beads was observed under light microscope (Figure 13).

Crude antigen preparation (Zymolyase extract, 1 mg/ml) was applied to, and subsequently eluted from columns in solutions with various ionic strength at different pH values.

A list of these buffers is shown in Table 1. Eluate was concentrated with negative pressure dialysis against the loading buffer (Table 1). Eluate was tested for the presence of antigen by capillary tube precipitation test against the 10G hybridoma culture fluid.



**Figure 13.** Attachment of *C. albicans* yeast cells on Mab-conjugated Sepharose-4B beads.

**Table 1.** Buffers for immuno-affinity chromatography.

| Loading buffers                               | Eluting buffers                    |
|-----------------------------------------------|------------------------------------|
| 0.01 M PB, 0.5 N NaCl, 0.05% Tween 20, pH 7.2 | 0.01 M PB, 2.0 N NaCl, pH 7.2      |
| 0.01 M PB, 0.5 N NaCl pH 7.2                  | 0.01 M PB, 4.0 N NaCl, pH 7.2      |
| 0.01 M PB, 0.15 N NaCl pH 7.2                 | 0.01 M PB, 0.5 M D-mannose, pH 7.2 |
|                                               | 0.01 M PB, 0.5 M D-glucose, pH 7.2 |
|                                               | 3.5 M Acetic buffer, pH 2.6        |

### Chemical Treatment of Crude Antigen Preparations.

Periodate. Yeast cells grown in GYEP at 37°C were washed three times in cold deionized water, suspended at  $2 \times 10^7$  cells/ml in 100 mM sodium periodate (Sigma) in 50 mM sodium acetate (Sigma), incubated at 4°C with shaking for 22 h and tested for agglutination with the monoclonal antibody previously described (Mab 10G, see Chapter 2). Solubilized crude antigen fractions (5 mg/ml) were also treated with periodate under the same conditions, neutralized with 100 mM ethylene glycol (Sigma) and tested for antigen activity by agglutination and SDS-PAGE-Western blot (see below).

Pronase B. Degradation of crude antigens by pronase B was done as described in Chapter 2 under the subtitle "Partial antigen characterization". Azocoll (Sigma) was used as a control substrate for the activity of the enzyme.

### SDS-PAGE and Western Blot Analysis.

Electrophoresis was performed in 10% or 7.5% acrylamide gels (30:0.8 by weight acrylamide:bisacrylamide) containing SDS as described by Laemmli (185). Samples were first denatured by heating to 65°C for 10 min in the presence of 4%  $\beta$ -mercaptoethanol and 2% SDS. Gels were run at a constant current of 25 mA in a vertical gel electrophoresis apparatus (Idea Scientific, Corvallis, OR). After electrophoresis, gels were stained in a solution of 0.05% Coomassie blue G-250

(Baker), 50% methanol, and 10% acetic acid, and destained by diffusion in a biphasic solution of 5% acetic acid and 1-butanol (9:1 v/v). In some experiments, periodate-acid-Schiff (PAS) stain was used to visualize polysaccharides (153).

For Western blot analysis, samples in the gel were electrophoretically transferred to nitrocellulose paper (pore size 0.2  $\mu$ m, Schleicher & Schuell, Keene, NH). Blotting was done at a constant voltage of 12 V (Genie blotter, Idea Scientific, Minneapolis, MN) for 4 h in Tris-glycine (Sigma) buffer (25 mM Tris-base, 192 mM glycine, pH 8.0) made in 20% methanol. The nitrocellulose sheet was stained with Ponceau S (0.5% Ponceau S [Sigma], 2.5% trichloroacetic acid, 2.5% acetic acid) for 2 min and then destained with 5% acetic acid until the transferred protein bands were visualized. The blot was blocked with 5% BSA in Tris-buffered saline with Tween-20 (TBST) (10 mM Tris-HCl, 0.15 N NaCl, 0.05% Tween-20) for 4 h at room temperature, washed three times for 30 min with TBST, and incubated with Mab 10G (1:100 diluted 10G ascites in TBST, titer = 2,400) (for ascites production, see Materials and Methods in Chapter 2) for 30 min. After washing three times for 10 min each in TBST, a secondary antibody (Sigma, goat anti-mouse whole IgG conjugated with alkaline phosphatase, diluted as manufacturer suggested) was added and incubated for 1 h at 24°C. The blot was washed three times for 10 min each in TBST and bands were detected

by reacting in substrate solution (0.1 M Tris-HCl, 0.15 N NaCl, 10 mM MgCl<sub>2</sub>, pH 9.5) containing 0.033% (w/v) nitroblue tetrazolium (NBT) and 0.017% (w/v) bromo-chloro-indolyl-phosphate (BCIP, Sigma). NBT and BCIP stock solutions were prepared at concentration of 50 mg/ml in 50% formamide.

#### Isolation of Epitope from Crude Antigen Extract.

Mannan Isolation. The solubilization of mannoproteins from crude extract was done according to procedures described elsewhere (199). Zymolyase extract (fraction II, 4 g) was dissolved in 100 ml of water and 50 ml of water containing 4 g of hexadecyltrimethylammonium bromide (cetavlon) was added. After standing at 24°C for 16 h, the precipitate formed was collected by centrifugation and washed with 50 ml of water. The supernatant and the water washing was combined for later procedures. The washed precipitate was dissolved in 1 M NaCl, precipitated with three times the volume of 95% ethanol, washed with 95% ethanol and dried *in vacuo*. This fraction was referred to as fraction II-A.

To the 200 ml of combined solution was added boric acid (100 ml) and the pH was adjusted to 8.8 with 2 N NaOH. After 16 h at 24°C, the precipitate which formed was centrifuged and washed with 0.5% sodium borate (pH 8.8). The supernatant from the centrifugation was saved for later procedures (final sup.). The washed precipitate was dissolved in 2% acetic acid (50 ml) to which 1 g of sodium acetate was added. Three

times the volume of 95% ethanol was then added to the solution, and the resulting precipitate was washed once in 2% acetic acid in 95% ethanol followed by an ethanol wash. The product was dried *in vacuo* and designated as fraction II-B.

The pH of the saved supernatant was adjusted to 9.5 with 2 N NaOH and allowed to stand for overnight at 24°C. The precipitate was washed with 0.5% sodium borate and isolated as described above. This fraction was referred to as fraction II-C.

Ethanol (95%, 3 volumes) was added to the remaining supernatant (final sup.), the resulting precipitate was washed in 2% acetic acid and 95% ethanol followed by an ethanol wash, and dried as described above, and designated as fraction II-D. The isolation procedures are summarized in Figure 14.

Latex Agglutination. Latex agglutination was used to detect soluble antigen in solution. Two percent solid latex beads (0.88  $\mu\text{m}$  sulfate polystyrene, Interfacial Dynamics Co. Portland, OR.) were mixed with an equal volume of HPLC-purified Mab 10G (200  $\mu\text{g}$  total protein/ml) in 0.01 M PBS. The suspension was mixed by rotation (RotoMix type 48200, Thermolyne Co. Dubuque, IA.) at 100 rpm at 24°C. After 12 h of mixing, BSA was added to a final concentration of 1%. The suspension was sonicated for 10 seconds (Biosonik IV, VWR Scientific) to disrupt the aggregates, and mixed for 1 h at

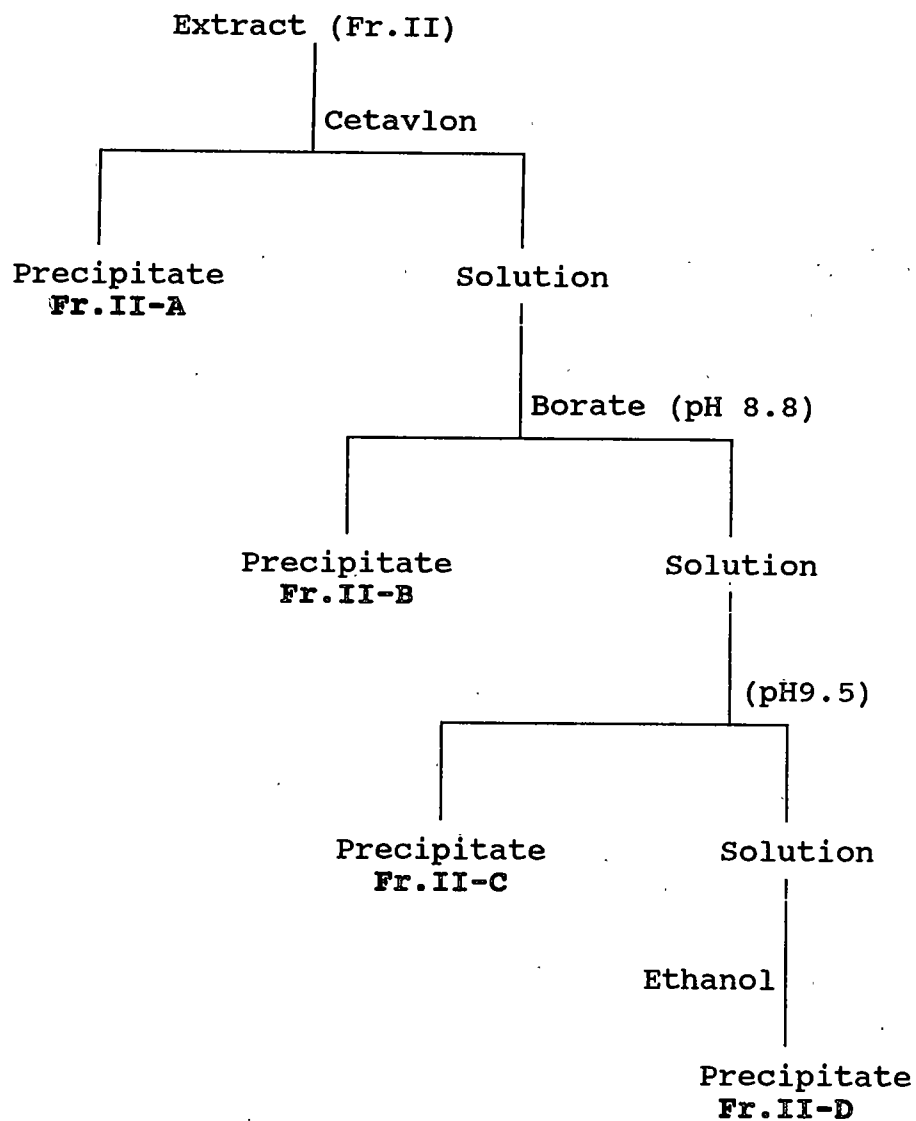


**Figure 14.** Scheme for mannoprotein isolation from Zymolyase extract (see text for details).

24°C. Beads were pelleted and resuspended to a concentration of 1% solid in PBS with 1% BSA and 0.02% sodium azide (Sigma). These latex beads conjugated with Mab 10G were referred to as latex-10G. Control beads were conjugated with BSA instead of Mab 10G (latex-BSA). In the agglutination

inhibition assay (see below), some latex beads were coated with rabbit anti-*C. albicans* polyclonal sera (latex-Pab) or a monoclonal antibody C6 which agglutinates *C. albicans* (28-30) (latex-C6), but is specific for a different antigen from the one recognized by Mab 10G. These beads were referred to as latex-C6. Agglutination assay was carried out by mixing 20  $\mu$ l of sample solution with 20  $\mu$ l of latex-10G on a ring slide (2 cm diameter ring, Scientific Products). The slide was gently rocked for 3-5 min and the agglutination (determined macroscopically and microscopically) was scored from +++, ++, +, to - as in the negative control.

Inhibition of Latex Agglutination. Latex-10G (10  $\mu$ l) were mixed with equal volume of sample at various concentration on a ring slide and rocked for 5 min. Twenty microliters of soluble antigen (fraction II) at various concentrations was added and mixed. After gently rocking the slide for 5 min, the degree of agglutination was scored. Control groups included latex-BSA, latex-C6, and latex-Pab. Several sugars of known compositions and structures were also used as specificity control. These includes stachyose, maltotetraose, and raffinose (Sigma).

Acid and Alkaline Degradation. Acid hydrolysis of mannoprotein was done following the procedure of others (304,305). Briefly, 100 mg of sample (Fr.II-B) was dissolved in 10 ml of 10 mM HCl (pH 2.0) and heated at 95°C (boiling)

for 60 min. The solution was cooled to room temperature, neutralized with 100 mM NaOH, and concentrated to 1 ml in a flash evaporator at 55°C. The resultant oligosaccharides mixture was fractionated on a column (1.5 by 165 cm) of Bio-Gel P-2 (400 mesh).

For alkaline degradation, 100 mg of sample was dissolved in 15 ml of 100 mM NaOH (pH 12.2) and the solution was left at room temperature for 18 h (249,304). After neutralizing with 1 M HCl, the solution was concentrated to 1 ml with flash evaporator and fractionated with P-2 column.

To confirm that  $\beta$  elimination had occurred under the alkaline condition, the formation of unsaturated hydroxyamino acids during the treatment of mannoprotein with 0.1 N NaOH was followed by measuring the absorbance at 240 nm (71).

Void volume and column calibration were determined by applying blue dextran (Sigma, MW 2,000 kd), sucrose and glucose. Fractions from the Bio-Gel P-2 column were eluted with water at a flow rate of 10 ml/h. Each fraction (50 drops, approximate 2.5 ml) was assayed for polysaccharide content with the phenol-sulfuric acid method described by Dubois (72). Briefly, 30  $\mu$ l of 5% phenol was added to 60  $\mu$ l of each fraction in a 96 well plate, 125  $\mu$ l of concentrated sulfuric acid was added to the center of the well to allow maximal mixing. The color reaction was monitored by a microtiter plate reader at 490 nm (Bio-Rad Labs, Richmond, CA). Fractions correspond to peak area were collected and

trough area were combined for re-chromatography. Void volume of acid-modified and alkaline-modified Fr.II-B were collected separately and designated as Fr.II-Ba and Fr.II-Bb respectively. Peaks were combined from several cycles of chromatography, concentrated by flash evaporator at 50°C, and tested for epitope presence by inhibition of latex agglutination.

#### Characterization of 10G Epitope.

Oligosaccharide fractions which inhibit latex-10G agglutination in the presence of high molecular weight antigen were further characterized with mass spectrometry and analyzed for sugar composition and glycosidic linkages.

Fast Atom Bombardment-Mass Spectrometry. Molecular mass of fractions isolated from P-2 column chromatography was determined by FAB-MS (VG 70E-HF). Glycerol was used as the matrix and accelerated xenon atoms (8 KeV, 1 mA primary current) caused sample ionization.

Proton Nuclear Magnetic Resonance (H-NMR). NMR spectra of oligosaccharide were recorded using a 500 MHz spectrometer. Samples were solubilized in D<sub>2</sub>O (0.4%) and analyzed at 30°C and 70°C respectively. Acetone ( $\delta=2.20$ ) was used as the internal standard.

Trimethylsilyl (TMS) Derivatives. Lyophilized samples (approximately 250  $\mu$ g) with 18  $\mu$ g of myo-inositol (internal

standard) were methanolized in 350  $\mu$ l of 1 M methanolic-HCl at 80°C for 16 h in Teflon capped tubes. Various sugars (100  $\mu$ g each, external standard) and inositol (added into the sample as internal standard) were also included. After incubation, five drops of tert-butanol (Baker) were added and the solution was evaporated at 40°C to dryness in nitrogen flow. In some samples, where amino-sugars were suspected, N-acetylation was carried out at this stage. To the dried samples, 300  $\mu$ l of methanol, 30  $\mu$ l pyridine, and 30  $\mu$ l acetic anhydride were added, mixed well, and incubated at room temperature for 6 h with caps tightly closed. Samples were evaporated under N<sub>2</sub> gas to dryness. Several drops (4-6) of toluene were added to help evaporation.

Methanolized and methanolized-N-acetylated samples were derivatized by adding 100  $\mu$ l of Tri-Sil reagent (a mixture of hexamethyldisilazine and trimethylchlorosilane and pyridine, Pierce) to each tube. Tubes were incubated at 80°C for 20 min. Excess Tri-Sil reagent was removed by passing filtered air over the sample solution in the tubes for 3 min at 24°C. Derivatized sugars were dissolved in 1ml hexane and tubes were centrifuged to remove precipitant. After the hexane was evaporated by filtered air, 50  $\mu$ l of hexane was added to dissolve derivatized sugars for GC analysis.

One microliter fractions of all samples were analyzed in a capillary column (DB-1, 30 M x 0.25 mm, J & W Scientific) in a Hewlett Packard 5890 GC equipped with a flame-ionization

detector. The chromatograph started at isothermal 160°C for 2 min, followed by a 2°C/min slope to 200°C, and then 10°C/min to 260°C and held isothermal for 5 min.

Alditol Acetate Derivatives. Lyophilized samples were hydrolyzed in 250  $\mu$ l of 2N trifluoroacetic acid (TFA) at 121°C for 1 h. Sample solution was evaporated by filtered air. 10 drops of iso-propanol were added to help evaporation. Hydrolyzed sugars were reduced to open chain form by addition of 300  $\mu$ l of 1% sodium borodeuteride ( $\text{NaBD}_4$ , Aldrich) in 1 M ammonium hydroxide for a minimum of 1 h at room temperature. The solution was neutralized by adding dropwise of glacial acetic acid until the bubbling ceases. Five hundred microliters of methanol-acetic acid mixture (9:1, v/v) was added and evaporate under  $\text{N}_2$  under room temperature. The addition and evaporation of methanol-acetic acid were repeated three more times before addition and evaporation of 0.5 ml of methanol (100%) for three times to complete dryness each time.

Reduced-dry samples were O-acetylated by the addition of 100  $\mu$ l of pyridine and 100  $\mu$ l of acetic anhydride at 121°C for 20 min with caps tightly closed. Samples were dry by filtered air after incubation. Two milliliters of methylene chloride and 1 ml of water were added, vortexed, and centrifuged for 15 min to separate two phases. The water phase was removed, and some sodium sulfate was added to

absorb water. The suspension was filtered over glass fiber-stuffed Pasteur pipette and evaporated under air to dryness. Samples were dissolved in acetone for GC analysis. An internal standard, myo-inositol, was included in each sample, and external sugar standards were prepared and treated the same way as the samples.

The GC column for alditol acetate derivatives analysis was a fused silica capillary column (SP-2330, 15 M x 0.25 mm, 0.2  $\mu$ m film thickness, Supelco) and a chromatograph programmed at 190°C isothermal for 3 min, 10°C/min slope to 240°C, and held isothermal for 10 min.

Linkage Analysis. Samples (500  $\mu$ g) in 0.5 ml DMSO were treated with 0.5 ml of 2.5 M butyllithium in hexane (Aldrich). The suspension was stirred with a magnetic stir bar for overnight at room temperature and then kept on ice bath for 20 min. One ml of iodomethane (Aldrich) was added to the suspension and keep stirring for a minimum of 4 h at room temperature. Two ml of water was added to stop reactions, and nitrogen gas was used to remove excess of iodomethane. The suspension was then passed through a Superclean-LC18 reverse phase column (Supelco) preconditioned with acetonitrile and water under negative pressure. Samples were eluted with 2.5 ml of acetonitrile and evaporated with N<sub>2</sub> until dryness. Methylated samples together with 18  $\mu$ g of myo-inositol (internal standard) were then hydrolyzed with

TFA, reduced with sodium borodeuteride, and O-acetylated as described for alditol acetate derivatization.

An alternative method was employed for the determination of reducing-terminal sugar. In this procedure, oligosaccharides were first reduced in 1% sodium borodeuteride in 1 M ammonium hydroxide for 2 h at room temperature. The solution was then neutralized with acetic acid. Borate was removed with the addition and evaporation of methanol-acetic acid mixture (9:1 v/v, three times) followed by three times addition and evaporation of absolute methanol. Dry sample was dissolved in water and the solution was passed through a Dowex 50W ion-exchange column to remove sodium ion. Sample was freeze-dried, dissolved in DMSO, and then methylated as described above.

Permethylated alditol acetate derivatives were injected into a capillary column (SP-2330, 30 M x 0.25 mm, 0.2  $\mu$ m film thickness, Spelco) in a HP-5970 GC-MS set up for electron impact ionization. Temperatures for GC separation of sugar derivatives was programmed at 80°C for 2 min, followed by 30°C/min to 170°C, held for 2 min, followed by 4°C/min slope to 240°C.

#### Immuno-Electron Microscopy.

Freeze-substitution methods for the fixation of exponential phase yeasts was described previously (198) (see also Chapter 2). Grids with the ultra-thin sections (60-90

nm) were incubated with 3% BSA in PBS for 10 min. Grids were then inverted and floated on 30  $\mu$ l of concentrated 10G hybridoma culture fluid pre-mixed with various concentration of fractionated epitope (1 mg/ml, 500  $\mu$ g/ml, and 250  $\mu$ g/ml final concentration) for 1.5 h. Some grids were incubated with culture fluid mixed with PBS or culture fluid from a non-secreting myeloma cell line. Grids were then incubated 2 h in 3% BSA, allowed to react with colloidal gold conjugated goat anti-mouse IgG+IgM (10 nm, Janssen Biotech.), and contrast-stained with lead citrate and uranyl acetate.

## Results

### Antigen Characterization.

SDS-PAGE and Western Blot Analysis. The aqueous phase phenol extract (see Chapter 2) and fraction I through IV were subjected to SDS-PAGE, Western blotting analysis and immunostaining. In 10% acrylamide gels, antigen in fraction I through IV barely migrated into the resolving gel and appeared as single high molecular weight bands (Figure 15a). However, when a lower concentration of gel was used (7.5%), antigen was found to be associated with smeared bands on top of the resolving gel (Figure 15b). In phenol extract, both high molecular weight smear and low molecular weight band were detected by immunostaining (Figure 15).

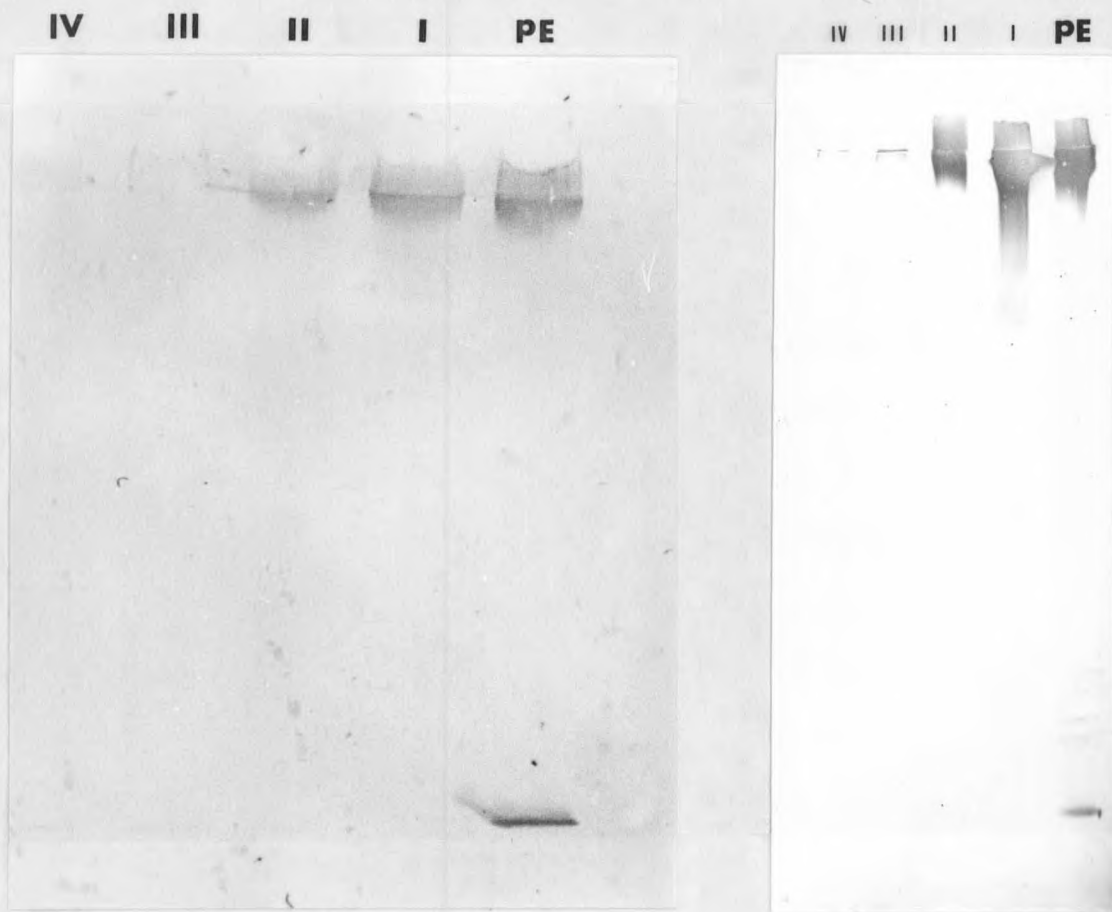


**Figure 15.** SDS-PAGE-Western blot analysis of antigens in phenol extract (PE) and Fr.I-IV. Samples were analyzed in 10% acrylamide gel (left) and 7.5% acrylamide gel (right).

When the gels (7.5% acrylamide) were stained with silver stain (for proteins) and Schiff reagent (for polysaccharides) (Figure 16), proteins with different molecular weight were observed among preparations. More protein bands were seen in Zymolyase extract than in other preparations. Carbohydrates were only found in the spacer gel and on top of the resolving gel (7.5%), correspond to the appearance of the antigen

(Figure 16 versus Figure 15).

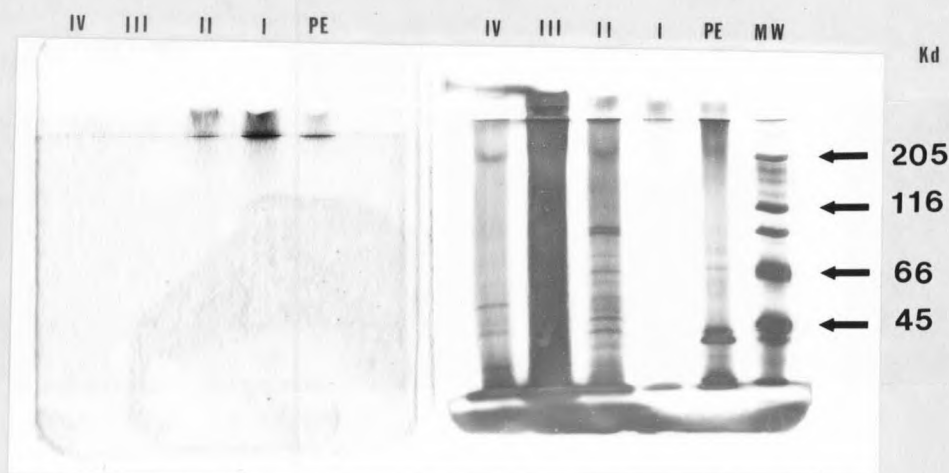


**Figure 16.** Silver stain (right) and PAS stain (left) of samples separated on SDS-PAGE. PE, phenol extract.

Treatment of antigen preparations with Pronase B (a non-specific protease) did not alter the agglutination titer with latex-10G (Table 2). However, no antigen was detected on the nitrocellular sheets when the treated preparations were subjected to SDS-PAGE and Western blotting.

Periodate Treatment. Whole yeast cells, after treated with 100 mM sodium periodate, were no longer agglutinated in the presence of Mab 10G. Antigens in phenol extract and fraction I through IV were also destroyed by sodium periodate treatment as demonstrated by the loss of agglutination with latex-10G (Table 2).

#### Immunoaffinity Chromatography.

Affinity chromatography was unsuccessful in the

purification of antigen from crude extracts. In all of the buffer systems used, no antigen was detectable in the eluate by capillary tube precipitation test.

Several reasons may explain the failure of the described method to purified antigen from the affinity column. First, the concentration of Mab adsorbed onto Sepharose beads was too low to give demonstratable isolation of the antigen. Second, the fact that Mab 10G belongs to the IgM class suggests a multi-valent binding site on the antibody. The affinity was amplified considering the high copy number of epitopes in carbohydrate side chains in the mannoproteins. Third, the assay used for detection of soluble antigen from column eluate involved the precipitation of antigen-antibody complexes, which requires an optimal proportion of each component. This method was probably less sensitive as compared with agglutination of Mab-coated latex beads. It is possible that a low concentration of antigen was eluted but beyond the detecting ability of the assay method.

In Chapter 4, we describe a successful method for the isolation of carbohydrate antigen from crude preparations.

#### Epitope Isolation and Characterization.

Mannoprotein Precipitation. Four preparations (Fr.II-A, B, C, and D) were obtained from Fr.II by pH-dependent precipitations in the presence of Cetavlon in boric acid. The agglutination titer of each preparation for latex-10G was

shown in Table 2. Because of the low antigenic activity in Fr.II-C and Fr.II-D, no further investigation was continued on these two fractions. Although Fr.II-A has a high antigenic activity, it was not very soluble in water at the concentration needed for further treatment. Therefore, Fr.II-B was used for further epitope isolation. Approximately 1 g of Fr.II-B was obtained from 4 g of Fr.II.

**Table 2.** Lowest concentrations of various extracts to agglutinate latex-10G beads.

| Fr.                | Conc.           | Fr.   | Conc.          |
|--------------------|-----------------|-------|----------------|
| I                  | 1 $\mu$ g/ml    | II-A  | 500 ng/ml      |
| I(SP) <sup>a</sup> | NA <sup>c</sup> | II-B  | 2 $\mu$ g/ml   |
| I(PB) <sup>b</sup> | 1 $\mu$ g/ml    | II-C  | 600 $\mu$ g/ml |
| II                 | 1 $\mu$ g/ml    | II-D  | 1 mg/ml        |
| II(SP)             | NA              | II-Ba | 0.5 mg/ml      |
| II(PB)             | 1 $\mu$ g/ml    | II-Bb | 5 $\mu$ g/ml   |
| III                | 50 $\mu$ g/ml   |       |                |
| III(SP)            | NA              |       |                |
| III(PB)            | 50 $\mu$ g/ml   |       |                |
| IV                 | 50 $\mu$ g/ml   |       |                |
| IV(SP)             | NA              |       |                |
| IV(PB)             | 50 $\mu$ g/ml   |       |                |

<sup>a</sup>, sodium periodate treatment; <sup>b</sup>, Pronase B treatment; <sup>c</sup>, no agglutination at concentration as high as 10 mg/ml.

Gel Filtration Chromatography. The eluting profiles of P-2 column of acid and alkaline degraded Fr.II-B (100 mg each) are shown in Figure 17. Fractions correspond to carbohydrate peaks as revealed by the phenol-sulfuric acid assay were collected. Some of the smaller peaks that eluted first were combined as peak I. Every peak, including the

void volume materials, was tested for agglutination with the latex-10G or for inhibition of agglutination.

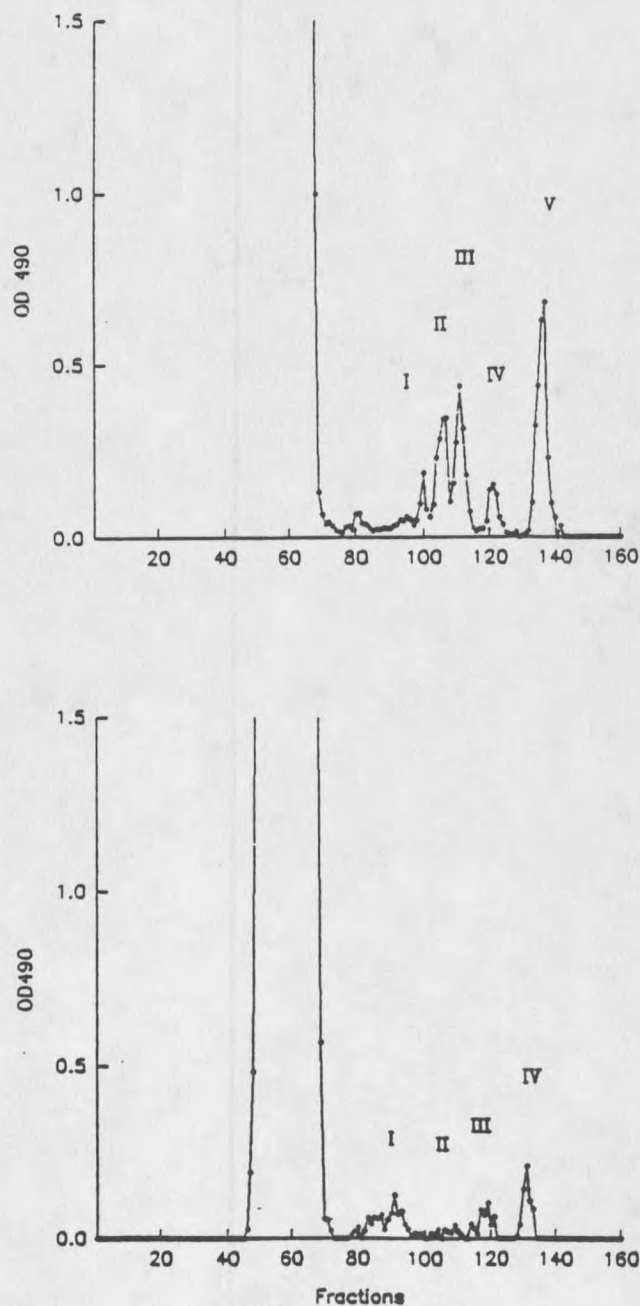


**Figure 17.** Eluting profiles of P-2 column chromatography of acid (a) and alkaline (b) treated Fr.II-B. Carbohydrate peaks were determined by phenol-sulfuric acid method.

Latex Agglutination. Rosettes of heat-killed *C. albicans* yeast cells by latex-10G are shown in Figure 18. All latex beads (latex-10G, latex-C6, and latex-polyclonal sera) were agglutinated in the presence of soluble antigens except latex-BSA. The agglutination titer of the void volume materials from chromatography on the P-2 column is shown in Table 2. Void volume material from alkaline degraded mannoprotein (Fr.II-Bb) has the same agglutination titer as compared to untreated mannoproteins (Fr.II-B). Acid hydrolysis, on the other hand, reduced the agglutinative activity by at least 100 fold (Fr.II-Ba).

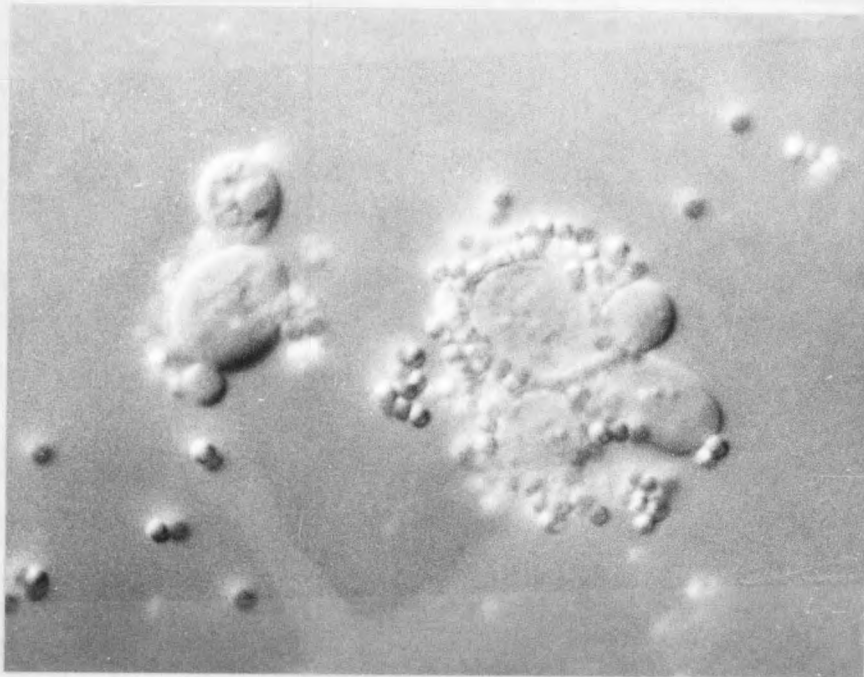


**Figure 18.** Rosette of *C. albicans* by latex-10G. Latex beads conjugated with Mab 10G were mixed with an equal volume of  $1 \times 10^8$  heat-killed *C. albicans* yeast cells. Note the beads on the surface of yeast cells.

Inhibition of Latex Agglutination. Oligosaccharide peaks from the P-2 column were tested for their ability to inhibit agglutination of latex-10G by soluble crude extracts. None of the alkaline degradation products inhibits latex agglutination. Only peak II from the acid-treated Fr. II-B was able to inhibit agglutination of latex-10G. Agglutination of other latex beads (latex-C6, latex-Pab) was not affected by this peak material. None of the standard sugars (raffinose, stachyose, and maltotetraose) showed inhibitory activity against latex agglutination.

The inhibitory activity of peak II against latex-10G agglutination was also concentration-dependent. The lowest concentration of soluble antigen needed to agglutinate latex-10G increased when sugar concentration in peak II was increased (as determined by phenol-sulfuric acid assay) (Table 3). Peak II and the next peak (peak III) were analyzed for molecular mass and sugar composition.

Peak I, the combined smaller peaks before peak II, caused direct agglutination latex-10G beads.

FAB-MS. FAB-MS showed that the molecular mass of peaks II and III were 666 and 504, respectively (Figure 19). These numbers correspond to a tetrahexose and trihexose, respectively.

Sugar Composition and Linkage Analysis. TMS and alditol acetate derivatives of P-2 fractions of acid-hydrolyzed

mannan preparation indicated that mannose was the only sugar in these oligosaccharides (peak II and III) (Figure 22, 23). When immunopurified 10G antigen (see Chapter 4) was analyzed, mannose was also found to be the major sugar with trace amount of galactose (Figure 24). The water extract of spheroplasts (Fr.III) showed mannose and glucose (Figure 25).

**Table 3.** Lowest concentration of antigen required to agglutinate latex-10G in the presence of peak II material.

| Sugar Conc. of Peak<br>II material | Soluble Antigen<br>(Fr.II) |
|------------------------------------|----------------------------|
| 2 mg/ml                            | 1 mg/ml                    |
| 1 mg/ml                            | 500 $\mu$ g/ml             |
| 250 $\mu$ g/ml                     | 250 $\mu$ g/ml             |
| 500 $\mu$ g/ml                     | 80 $\mu$ g/ml              |
| 125 $\mu$ g/ml                     | 40 $\mu$ g/ml              |
| 60 $\mu$ g/ml                      | 20 $\mu$ g/ml              |

Latex-10G beads were mixed with peak II of various sugar concentrations (left panel). After mixing for 5 min, soluble antigen at various concentration was added. The lowest concentrations of the soluble antigen required to agglutinate the latex beads were recorded (right).

Gas chromatography (GC) of permethylated alditol acetate (PMAA) derivatives of peak II revealed two major components (Figure 26). Fragmentation patterns of electron impact-mass spectroscopy (MS) showed these two components to be terminal mannopyranose (2,3,4,6-tetra-O-methyl-1,5-di-O-acetylmannitol) and 2-linked mannopyranose (3,4,6-tri-O-methyl-1,2,5-tri-O-acetyl-mannitol) (Figure 27). In order to determine the reducing-terminal sugar, samples were pre-reduced and subsequently permethylated and acetylated for GC-

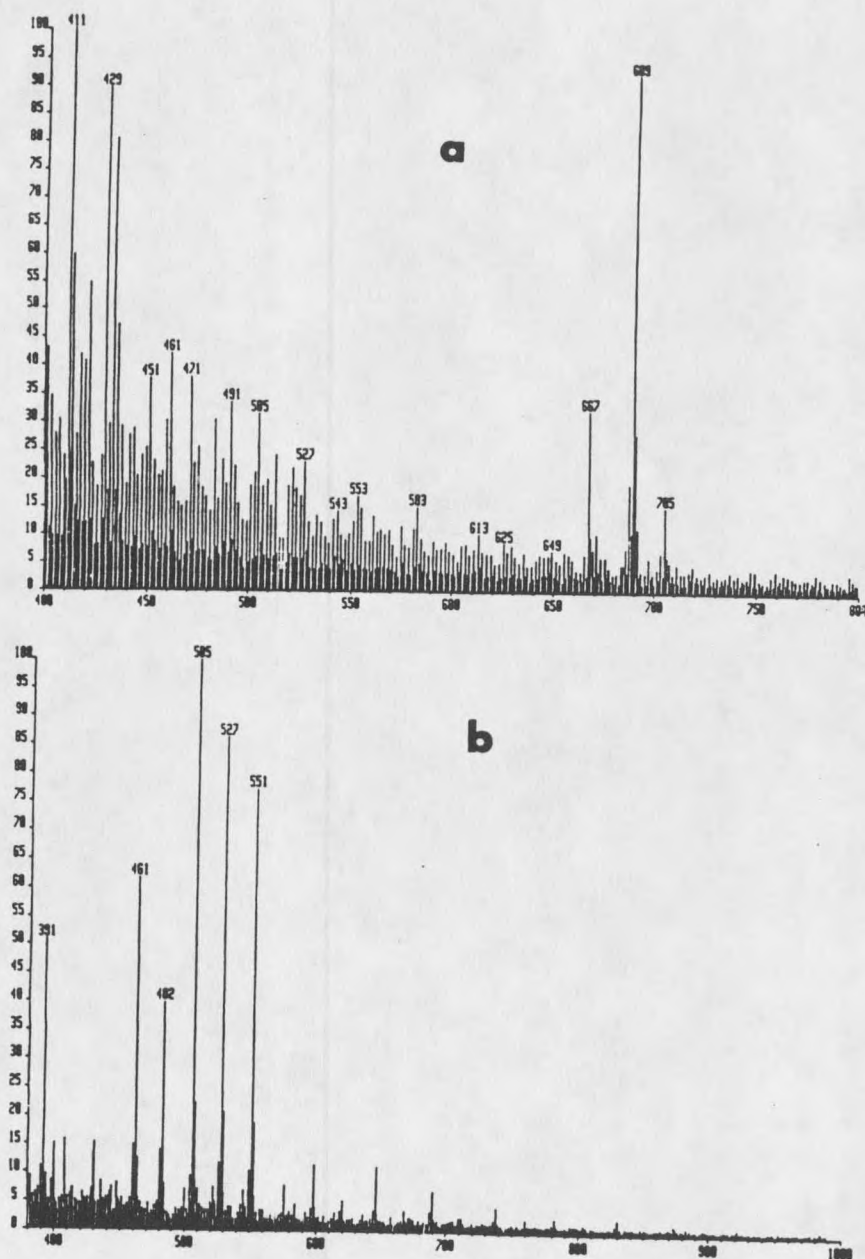
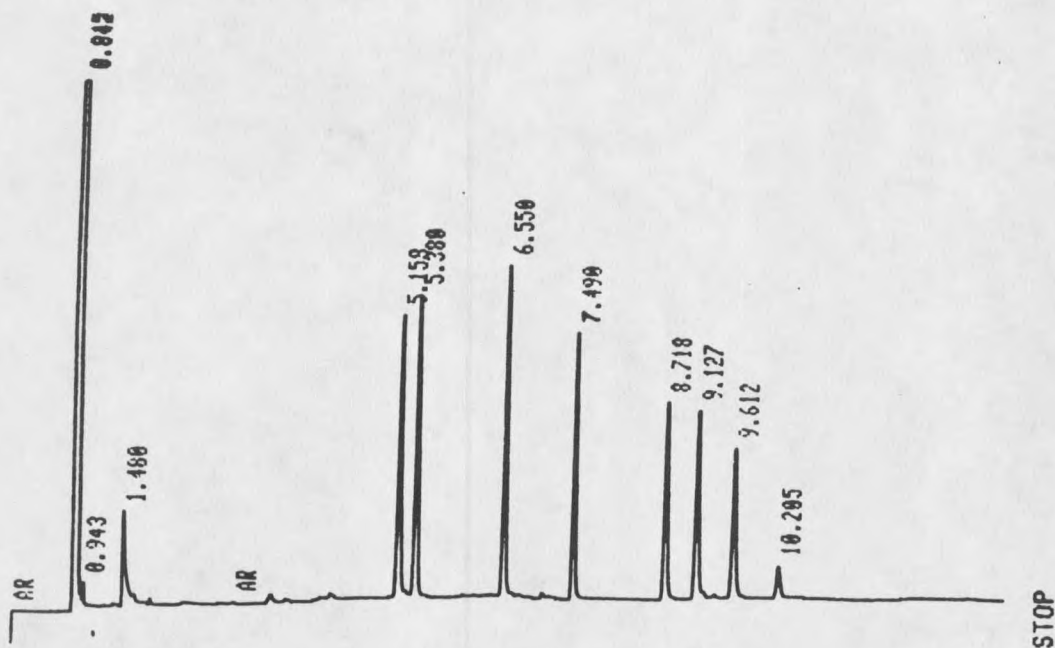


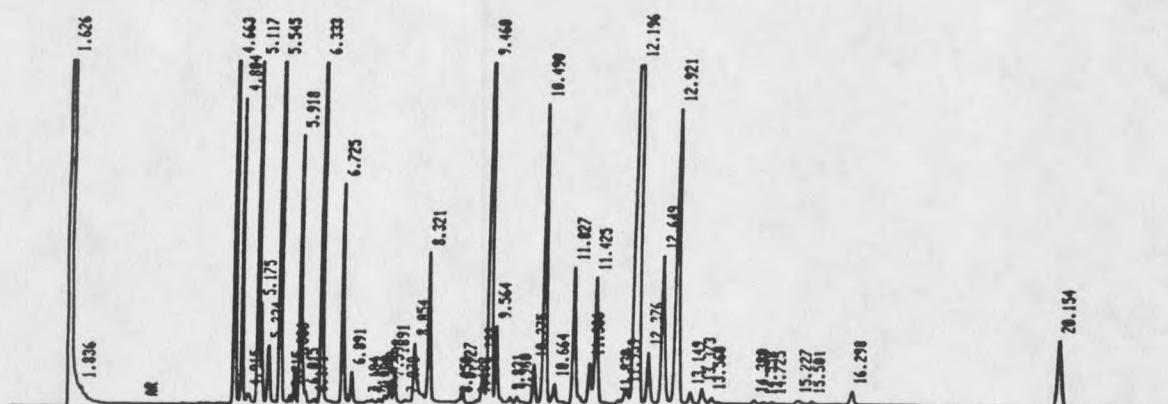
**Figure 19.** FAB-MS of peak II (a) and peak III (b) materials eluted from P-2 column. (a) Peaks at  $m/e$  667, 689, and 705 correspond to  $M+H$  (1),  $M+Na$  (23), and  $M+K$  (39), respectively. (b) Peaks at  $m/e$  505 and 527 represent  $M+H$  and  $M+Na$ , respectively. Atomic masses of peak II and III are 666 and 504, respectively.



| Retention Time (min) | % Area | Sugars      |
|----------------------|--------|-------------|
| 5.158                | 15.58  | L-Rhamnose  |
| 5.380                | 17.07  | L-Fucose    |
| 6.55                 | 17.33  | D-Arabinose |
| 7.49                 | 13.42  | D-Xylose    |
| 8.718                | 11.67  | D-Mannose   |
| 9.127                | 12.09  | D-Galactose |
| 9.612                | 10.53  | D-Glucose   |
| 10.205               | 2.29   | Inositol    |

**Figure 20.** Gas chromatography of altitol acetate derivatives of standard sugars.

MS analysis. Three major components were evident from the GC separation (Figure 28). The existence of penta-methyl mannitol (1,3,4,5,6-penta-O-methyl-2-O-acetylmannitol) demonstrates that the reducing terminal mannose is also 2-linked (Figure 29). The peak area ratio between reducing



| Retention Time (min) | % Area | Sugars      |
|----------------------|--------|-------------|
| 4.663                | 7.51   | D-Arabinose |
| 4.804                | 3.17   | D-Arabinose |
| 5.117                | 5.7    | L-Rhamnose  |
| 5.175                | 1.00   | L-Fucose    |
| 5.324                | 1.06   | L-Rhamnose  |
| 5.545                | 6.79   | L-Fucose    |
| 5.918                | 3.57   | L-Fucose    |
| 6.333                | 6.22   | D-Xylose    |
| 6.725                | 2.91   | D-Xylose    |
| 8.05                 | 1.23   | Glu-A       |
| 8.321                | 2.17   | Gal-A       |
| 9.303                | 0.61   | Gal-A       |
| 9.46                 | 8.78   | D-Mannose   |
| 9.564                | 1.25   | D-Galactose |
| 10.275               | 0.69   | D-Mannose   |
| 10.49                | 5.5    | D-Galactose |
| 11.027               | 2.45   | Gal-A       |
| 11.306               | 0.76   | Gal-A       |
| 11.425               | 2.30   | D-Galactose |
| 12.196               | 14.8   | D-Glucose   |
| 12.376               | 0.94   | Glu-A       |
| 12.649               | 3.12   | Glu-A       |
| 12.921               | 6.23   | D-Glucose   |
| 20.154               | 1.62   | Inositol    |

**Figure 21.** Gas chromatography of TMS derivatives of standard sugars. Glu-A, glucuronic acid. Gal-A, galacturonic acid.

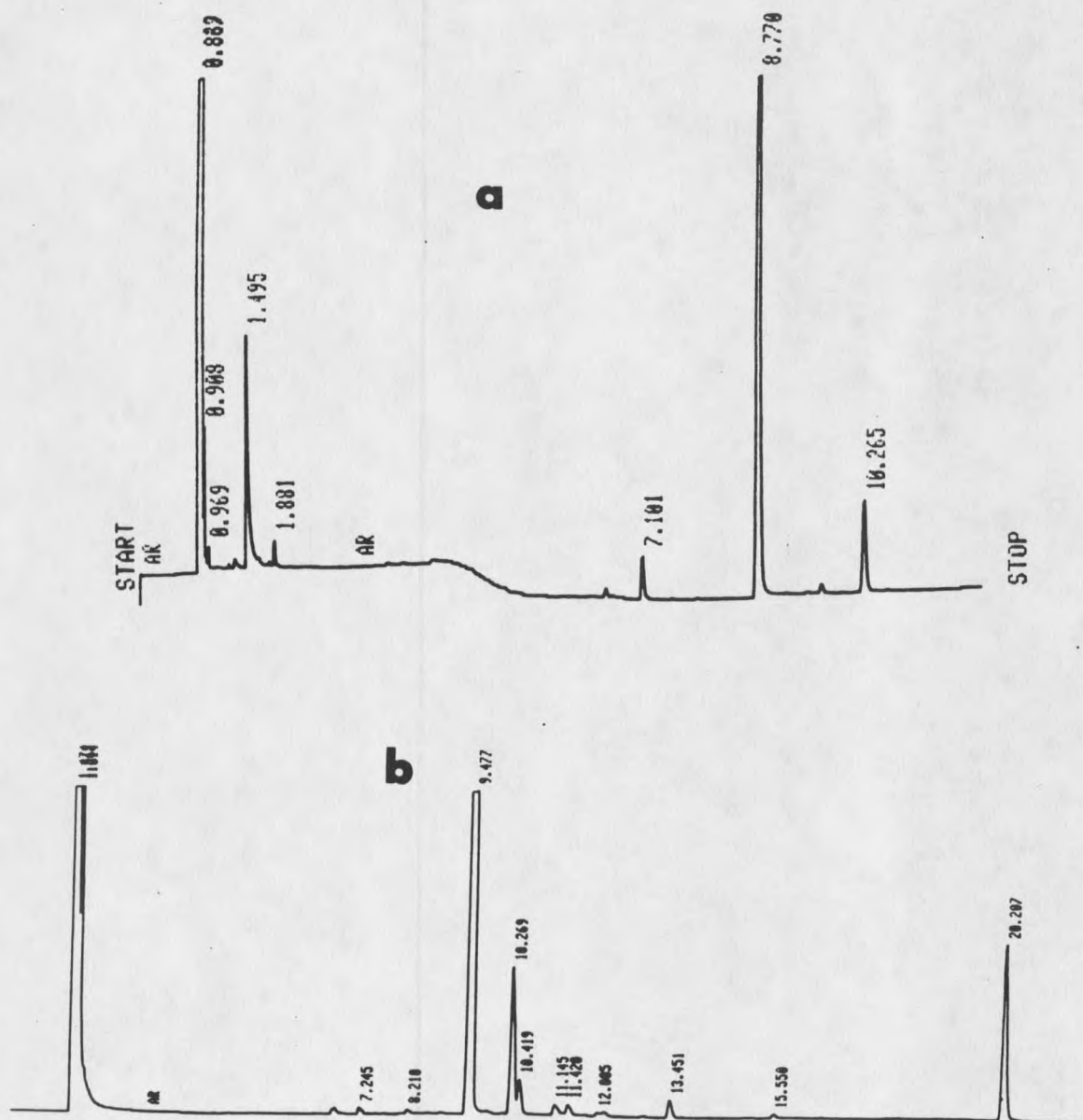


**Figure 22.** Gas chromatography of alditol acetate (a) and TMS (b) derivatives of peak II materials from P-2 column chromatography of acid-degraded Fr.II-B. (a) Peak of retention time 8.7 indicates D-mannose. (b) Peaks of retention time 9.47 and 10.26 also indicate the presence of D-mannose (compare to standard sugars in Figure 20 and 21).

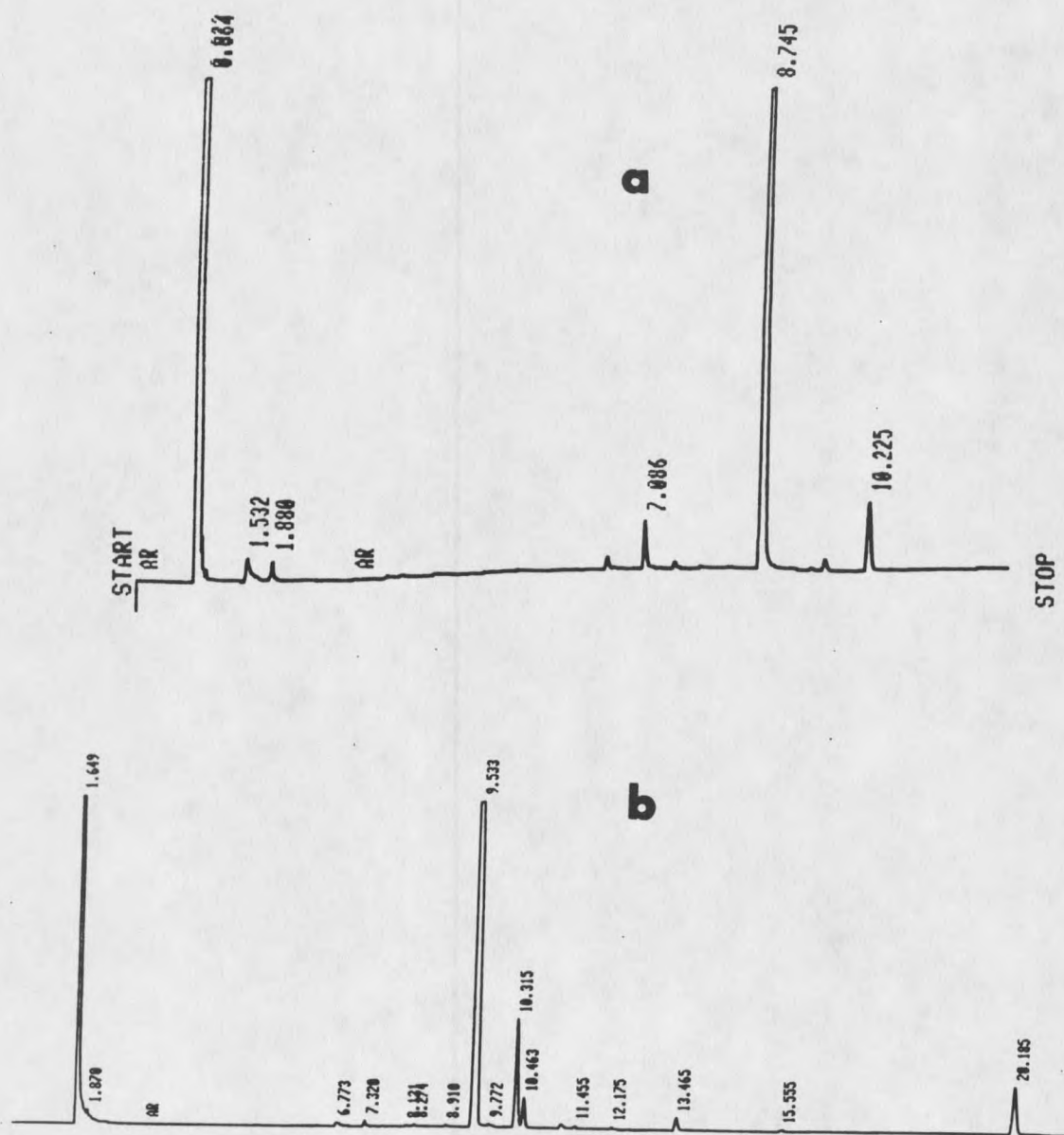


**Figure 23.** Gas chromatography of alditol acetate (a) and TMS (b) derivatives of peak III materials from P-2 column of acid-degraded Fr.II-B. (a) Peak of 8.7 min retention time represents D-mannose. (b) Peaks of 9.5 and 10.3 min retention time also indicate D-mannose (compare to sugar standard in Figure 20 and 21).

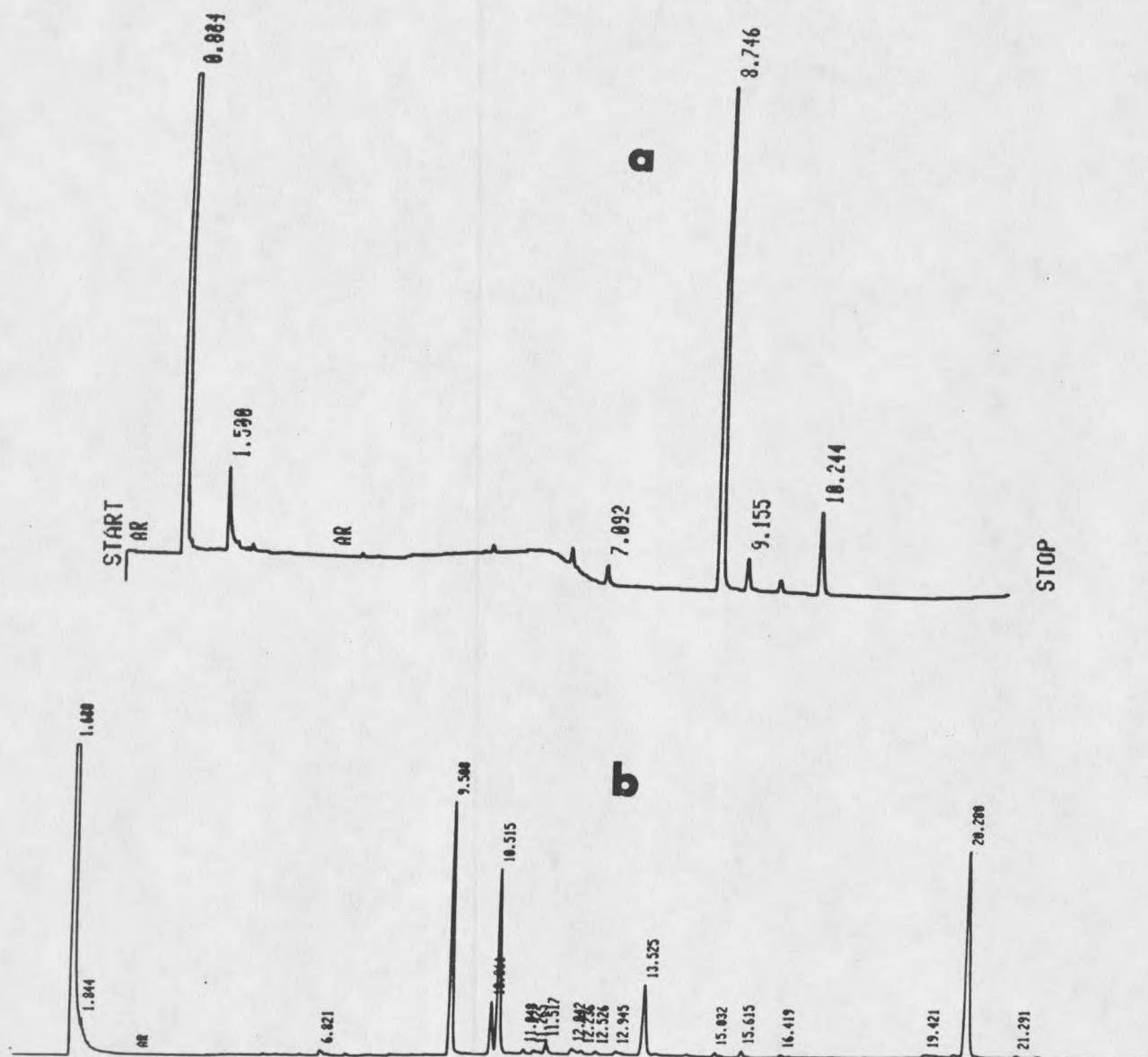


**Figure 24.** Gas chromatography of alditol acetate (a) and TMS (b) derivatives of immunoprecipitated 10G antigen. Mannose constitute the major sugar component in this preparation. Galactose was also observed (9.1 and 10.5 min retention time in (a) and (b), respectively).

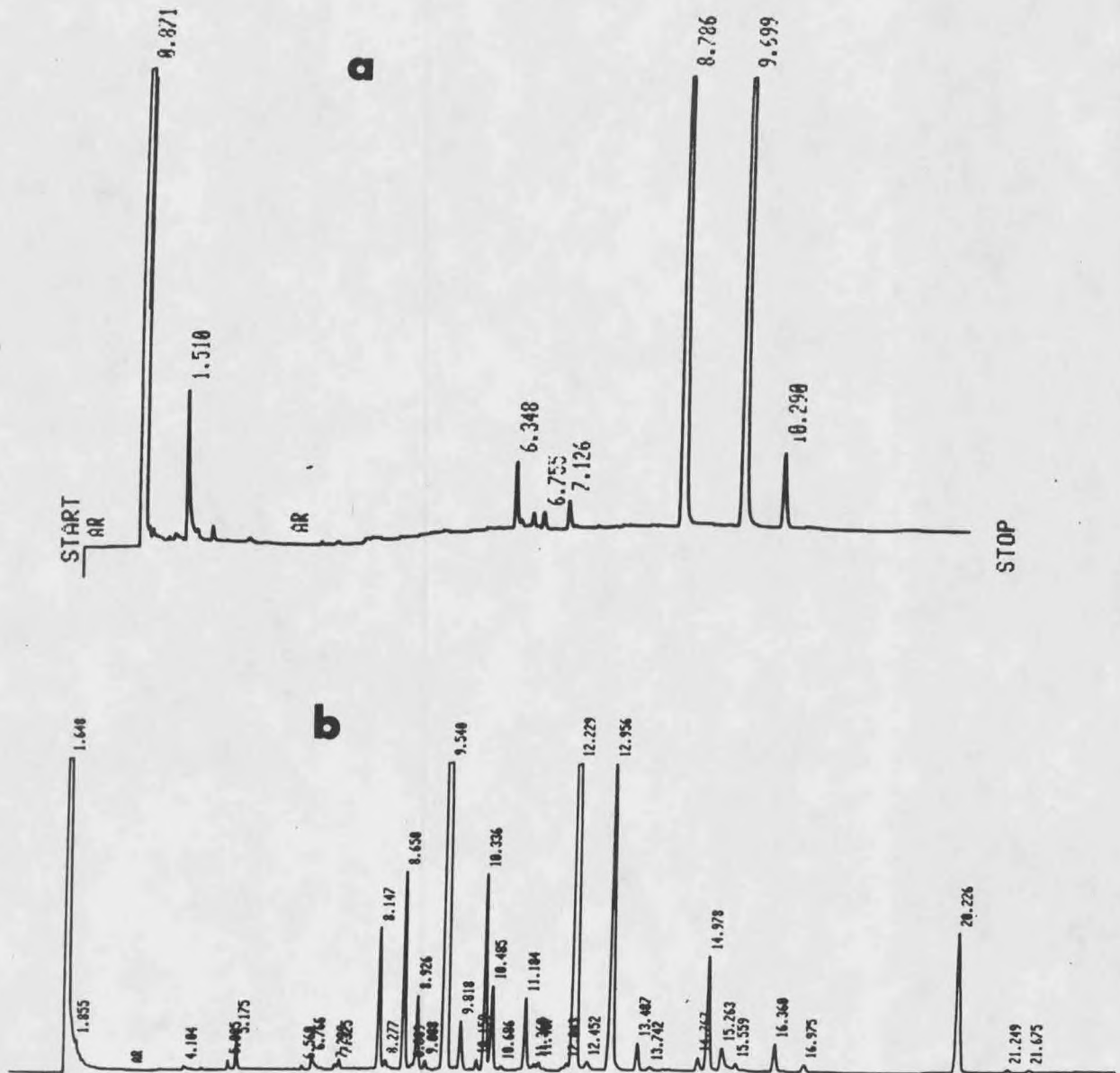


**Figure 25.** GC of alditol acetate (a) and TMS (b) derivatives of Fr.III. The presence of mannose and glucose were evident in both derivatization analysis.

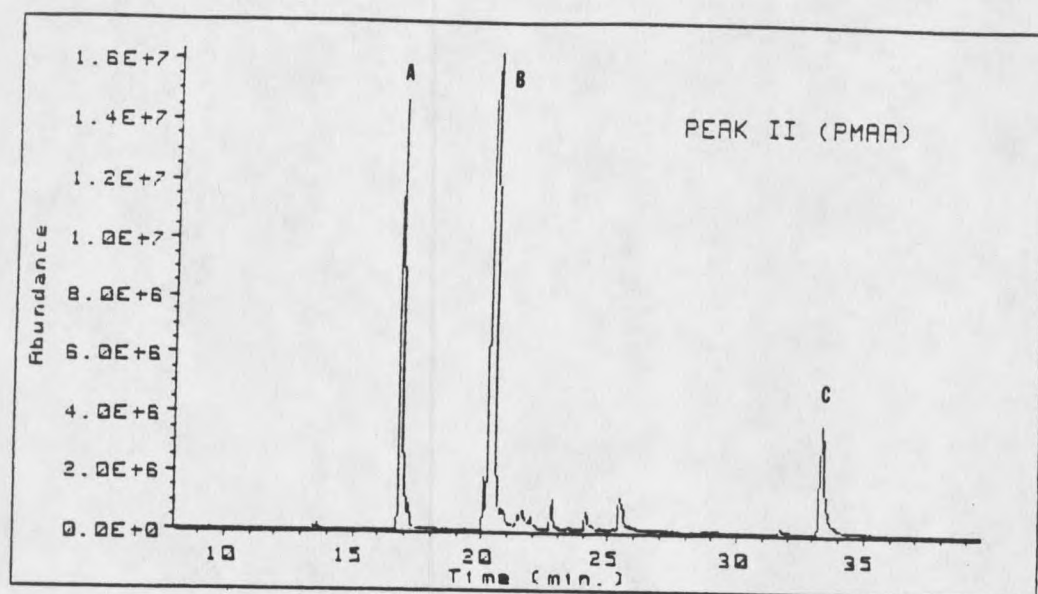


**Figure 26.** GC of PMAA derivatives of peak II. Two major components (A and B) were analyzed by electron-impact MS (Figure 27). (C) Inositol peak.

terminal, non-reducing terminal, and 2-linked mannitol was close to 1:1:2 (Figure 28), suggesting that the 1,2-linked tetramannose has a linear structure.

Peak III material was also analyzed by methylation for its linkage. The peak area ratio (2:1) of 2-linked mannopyranose to terminal-mannopyranose shows that this trihexose share the same structure as peak II but one mannose residue shorter (Figure 30).

Linkage analysis of immunopurified 10G antigen revealed a complex structure with major 2-linked mannopyranose and terminal mannopyranose peaks and several other minor peaks

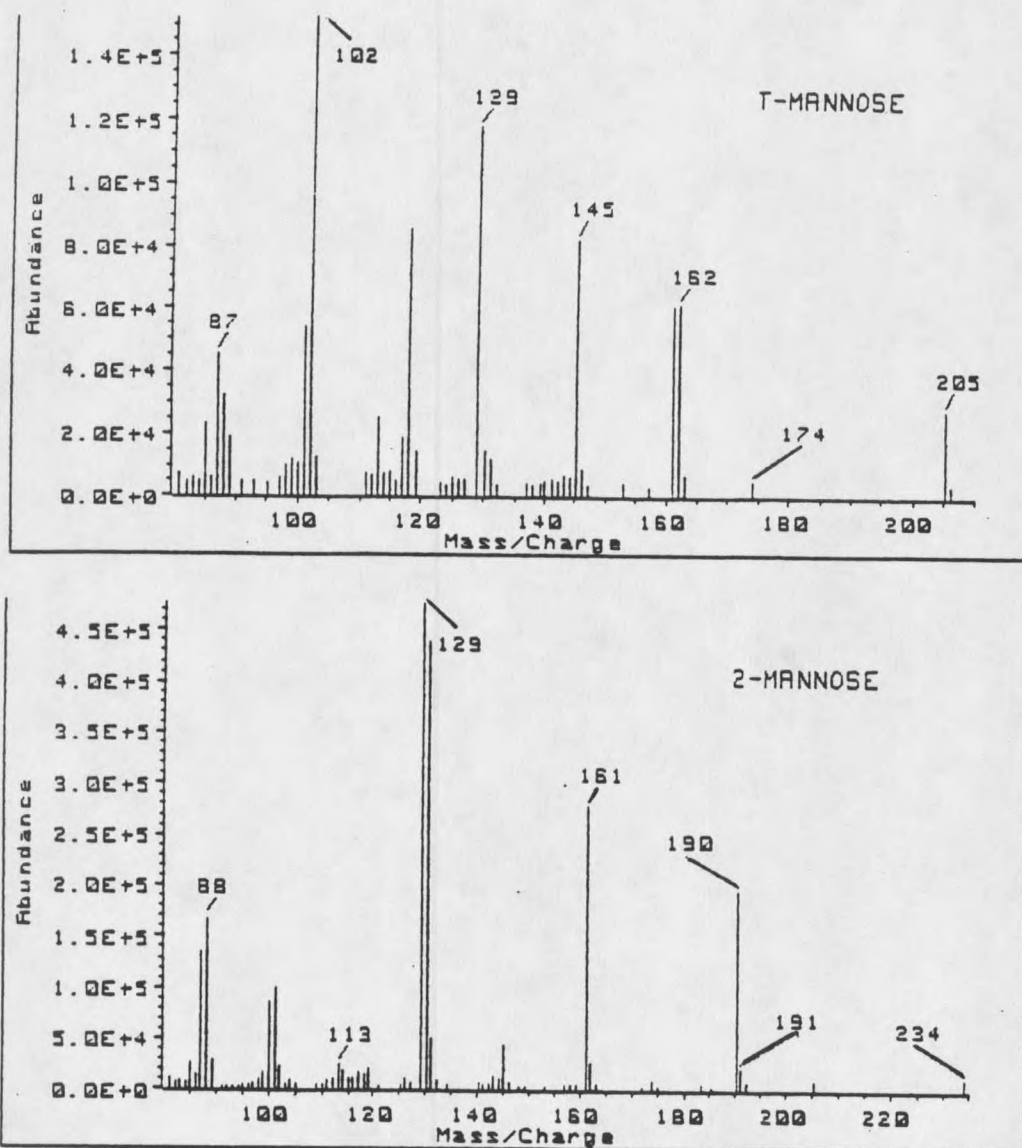
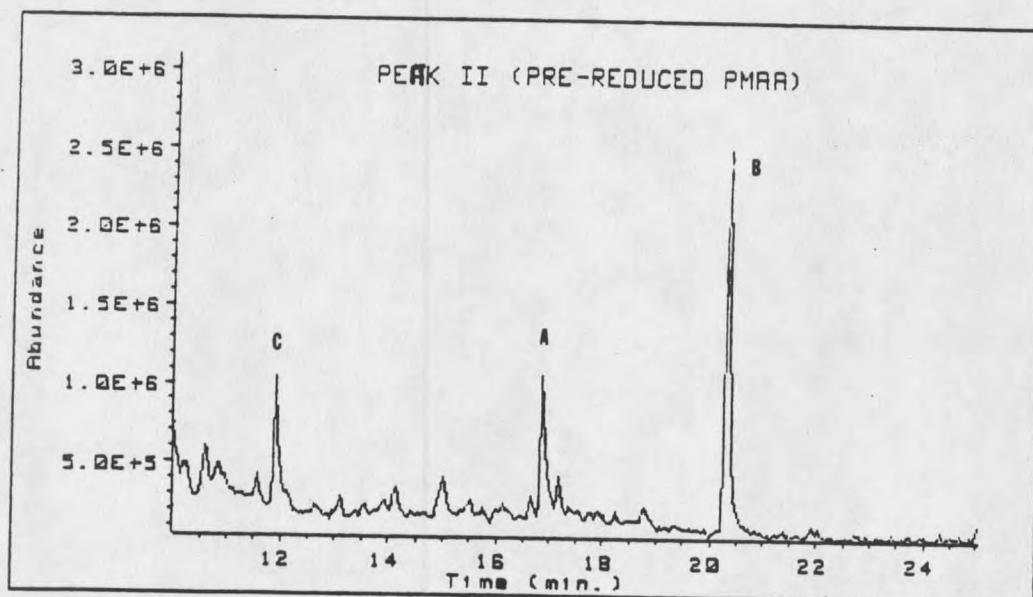


**Figure 27.** Fragmentation patterns of components A (upper) and B (lower) as indicated in Figure 26. In the upper panel, doublet of  $m/e$  at 161 and 162 indicates the fragmentation between two O-methylated carbons 3 and 4 (C-3, C-4). Peaks at 118 and 205 indicate breakage between two O-methylated C-2 and C-3. This pattern corresponds to a non-reducing terminal hexose. Lower panel, peaks at  $m/e$  161 and 190 indicate fragmentation between C-3 and C-4, both carbons are O-methylated. The pattern corresponds to a 2-linked hexose.

representing 6-linked and 2,6-branched mannopyranose (Figure 31 and Figure 32).



| Peaks | Retention Time (min) | Peak Area |
|-------|----------------------|-----------|
| C     | 11.906               | 64695194  |
| A     | 16.853               | 64532410  |
| B     | 20.302               | 166380931 |

**Figure 28.** GC of pre-reduced PMAA derivatives of peak II. The reducing-terminal was reduced before methylation and acetylation (see text for details). Peak area ratios between C, A, and B are close to 1:1:2. Mass spectroscopy analysis of A and B are the same as T-mannose and 2-mannose as shown Figure 27. Fragmentation pattern of component C is shown in Figure 29.

**NMR Spectra.** H-1 signals of proton NMR of peak II from P-2 column was recorded in a 500 MHz spectrometer at 70°C and 30°C (Figure 33). The acetone standard was set at 2.20 ppm. As indicated by Suzuki and coworkers (304,305,335), the signal at  $\delta$ 4.90 corresponds to the H-1 proton of  $\alpha$ -1,6-linked

mannopyranose and H-1 proton of  $\beta$ -1,2-linked non-terminal mannopyranose. Since no 1,6-linkage was observed in the methylation analysis, the signal at  $\delta 4.90$  indicate the  $\beta$ -configuration of H-1 proton of the two internal mannopyranose of the tetramannose. The signal at  $\delta 4.82$  corresponds to the non-reducing terminal H-1 proton in the  $\beta$ -1,2 linkage as described by Kobayashi et al (173). Signals at  $\delta 5.26$  and  $\delta 5.01$  correspond to the  $\beta$ - and  $\alpha$ -configuration, respectively, of the H-1 proton at the reducing end (173). The H-1 signal assignment is summarized in Table 4. The data obtained from NMR and methylation analysis indicate that the epitope contains  $\beta$ -1,2-linked linear tetramannosyl residue (Figure 34).

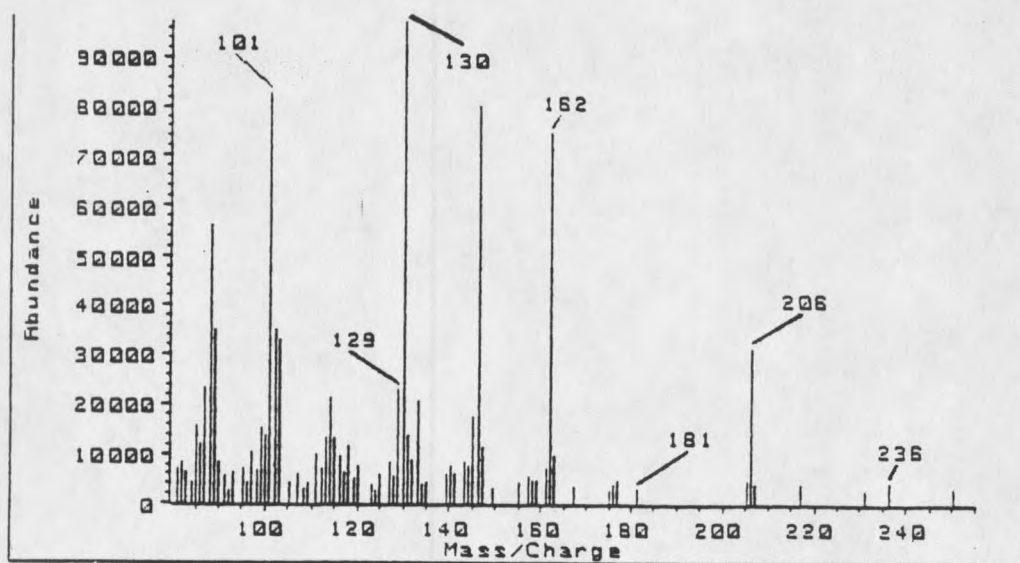
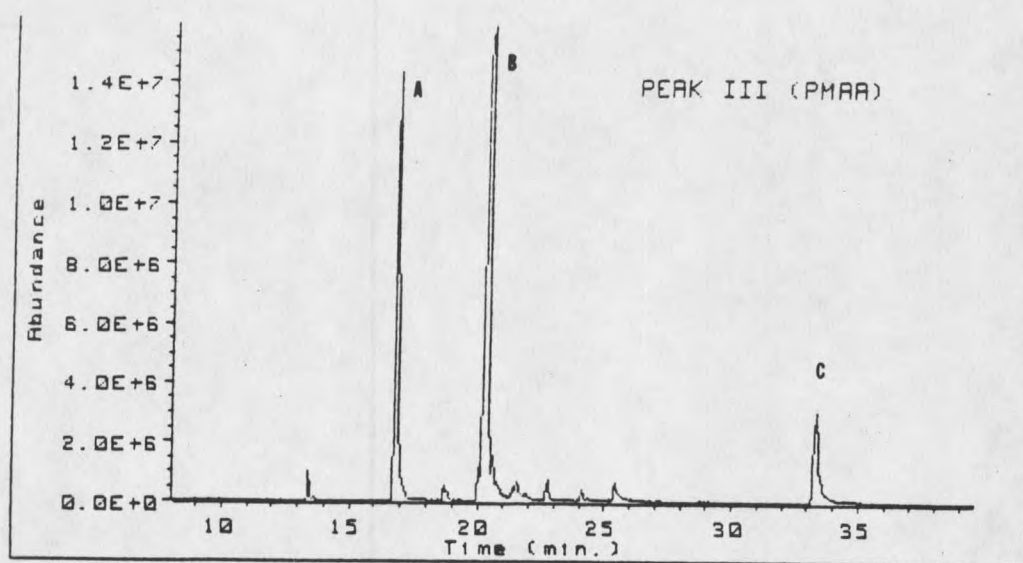


**Figure 29.** Fragmentation pattern of component C from Figure 28. Peaks of  $m/e$  at 206 and 162 indicate that fragmentations occurred between C-3, C-4, and C-4, C-5. Pattern shows that all carbons except C-2 are O-methylated. These information demonstrate that the reducing-terminal mannose is 2-linked.



| Peaks | Retention Time (min) | Peak Area  |
|-------|----------------------|------------|
| A     | 16.930               | 2133270133 |
| B     | 20.521               | 3555982532 |
| C     | 33.501               | 1258712303 |

**Figure 30.** GC of PMAA derivatives of peak III sugars. Peak area ratio between A and B is approximately 1:2. (C) Inositol peak. Fragmentation patterns of A and B are the same as T-mannose and 2-mannose, respectively (Figure 27).

#### Immuno-Electron Microscopy.

Sections stained with Mab 10G without the epitope revealed the surface and subsurface antigen expression (Figure 35). Staining with the Mab to both locations was blocked in the presence of purified epitope (peak II). At concentration of 1 mg/ml of epitope, labeling of gold particles onto the cell surface and plasma membrane were both inhibited (Figure 35a). Similar results were observed when 500  $\mu$ g/ml of epitope was used (Figure 35b). Gold labeling

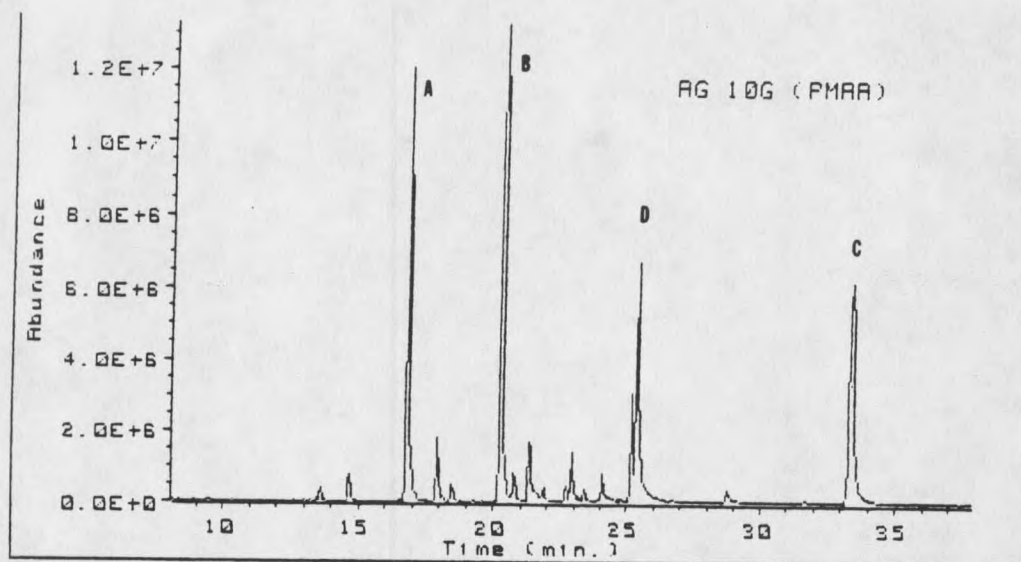


**Figure 31.** GC of PMAA derivatives of immunoprecipitated 10G antigen. Peaks A and B correspond to T-mannose and 2-linked mannose as shown in Figure 28. (C), Inositol. Fragmentation pattern of peak D is shown in Figure 32.

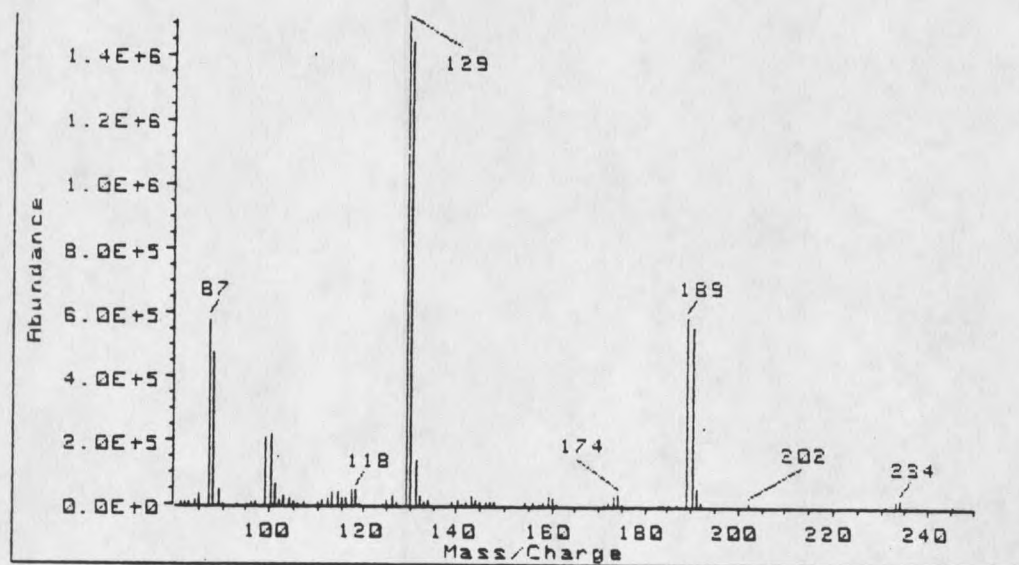


**Figure 32.** Fragmentation pattern of component D as shown in Figure 31. Doublet of  $m/e$  at 189 and 190 indicate fragmentation between methylated C-3 and C-4. All other carbons are O-acetylated, indicating a 2,6-branched linkage.

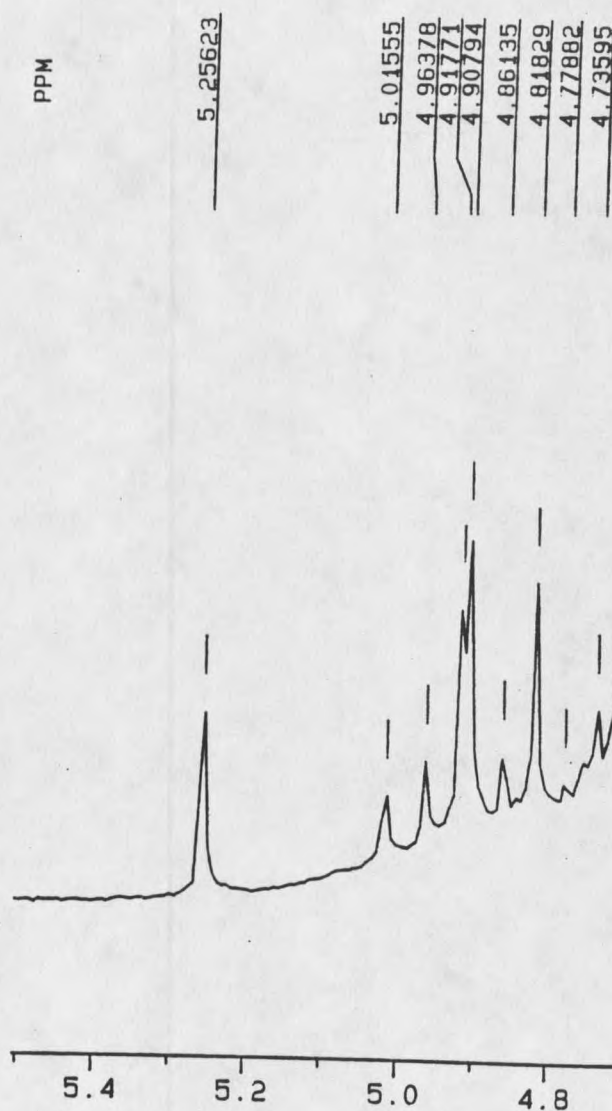


**Figure 33.** H-1 region (ppm) of proton-NMR spectrum of peak II material from P-2 column chromatography. Sample in D<sub>2</sub>O (0.4%) was analyzed at 70°C in a 500 MHz spectrometer using acetone ( $\delta=2.20$ ) as the internal standard.

re-appeared when the epitope concentration was dropped to 250  $\mu\text{g/ml}$  (Figure 35c). No gold particle was observed in the control group where sections were stained with culture fluid from a non-secreting cell line.

**Table 4.** H-1 chemical shift (ppm) of 1,2-linked linear tetramannosyl residues in either  $\alpha$  or  $\beta$  configuration (173,335).

| $M^a \rightarrow^2 M^b \rightarrow^2 M^c \rightarrow^2 M^d$ |                |      |      |                |               |
|-------------------------------------------------------------|----------------|------|------|----------------|---------------|
| anomeric<br>forms                                           | $\delta$ (ppm) |      |      |                |               |
|                                                             | a              | b    | c    | $d_{(\alpha)}$ | $d_{(\beta)}$ |
| $\alpha$                                                    | 5.05           | 5.26 | 5.27 | 5.34           | 4.89          |
| $\beta$                                                     | 4.82           | 4.90 | 4.91 | 5.27           | 5.01          |

M, D-mannose.

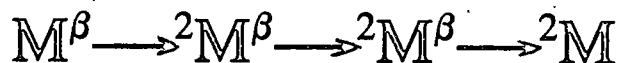


**Figure 34.** Structure of the epitope of Mab 10G. M, D-mannose.

### Discussion

We have previously reported that the antigen recognized by Mab 10G was resistant to heat and proteolytic enzyme treatment, thus suggesting that the antigen is a carbohydrate (198). We have also established that Zymolyase treatment in preparing spheroplasts does not disturb the antigen on the plasma membrane (198) (see also Chapter 2). In this report, antigens from the cell wall and plasma membranes were obtained by sequential fractionation. Similar electrophoretic patterns were observed between cell wall and membrane antigens. The antigens barely enter the separating gel, and appear to be carbohydrates as revealed by the PAS

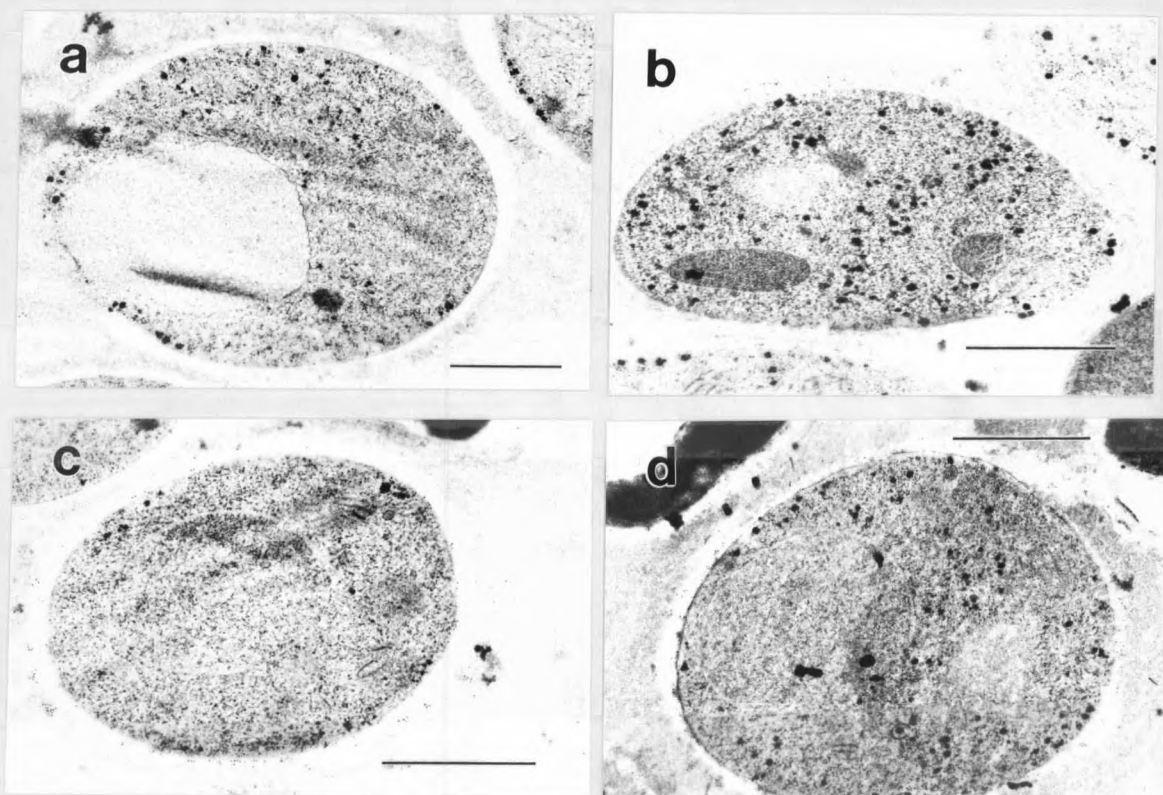


**Figure 35.** Inhibition of Mab 10G-colloidal gold labeling onto yeast cells by purified 10G epitope. (a, b, and c) Sections incubated with Mab in the presence of 1 mg/ml, 500 µg/ml, and 250 µg/ml of epitope, respectively. Note the rare appearance of colloidal gold particles in (a) and (b) as compared to (c) and (d). (d) Sections stained with Mab-10G without the epitope. Bars, 1 µm.

stain. Similar observation has been reported by others that a glycoprotein isolated from the cell wall of *C. albicans* resembled glycoproteins on the membrane surface in their electrophoretic characteristics (217). Treatment with Pronase B removed the protein moiety of the antigen and thus prevent the subsequent electrophoresis and electroblotting onto the nitrocellular paper. However, Pronase treatment did not affect the ability of the antigens to agglutinate latex

beads coated with Mab 10G. These data, together with the periodate-sensitivity of the antigen, suggest that the cell wall and membrane antigens are glycoproteins and Mab 10G is specific for a carbohydrate epitope.

Antigens expressed on the cell surface and plasma membranes of *C. albicans* have been reported by other investigators (134,257,267,330), and the membrane location is speculated to represent sites of mannan synthesis and secretion (267,330). However, my work is the first to characterize a specific antigen, as defined by a monoclonal antibody, and the structure of the epitope that reacts with the monoclonal antibody. My findings of the chemical structure of the epitope, which is a  $\beta$ -1,2-linked tetramannose, agree with work by Suzuki and colleagues who found that mild acid treatment of *C. albicans* mannans releases oligomannosyl residues of variable lengths which consist solely of  $\beta$ -1,2-linkages (173,305). It is thus likely that Mab 10G recognizes an antigenic determinant that is expressed on the acid-labile phosphomannose side chains of the mannan (Figure 36). The low agglutination titer of acid-modified mannoprotein (as compared to native mannoproteins) also support this deduction (Table 2).

It was suggested by others that cell wall mannoproteins are mostly distributed on the cell wall surface and inner layer next to the plasma membrane (301). My data suggest that the same structural determinant may be shared by

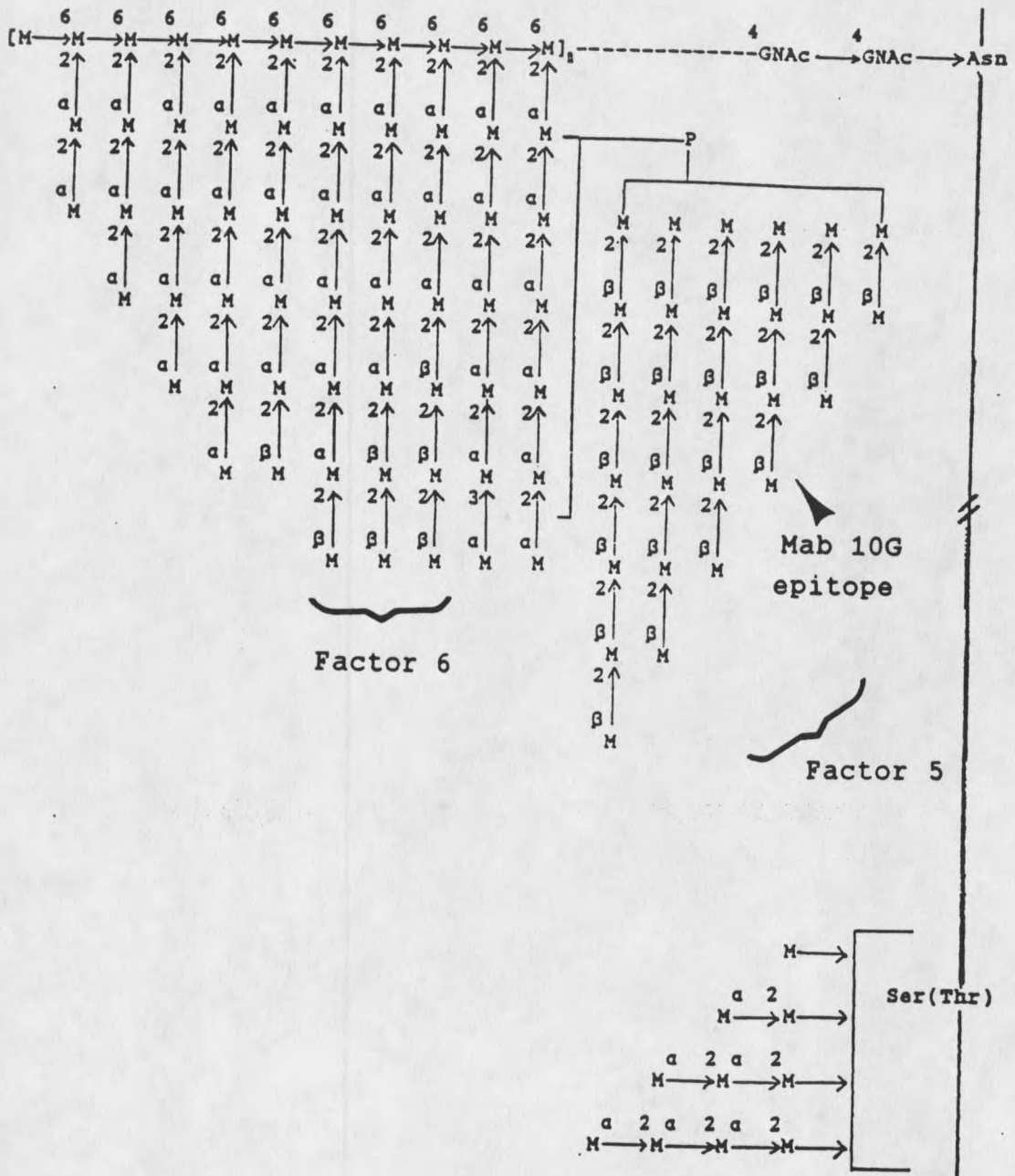


**Figure 36.** Structure of the 10G epitope relating to the proposed antigenic factor 6 and factor 5 on the cell wall mannan of *C. albicans*.

mannoproteins from both locations. Although the epitope was originally prepared from the Zymolyase extract, which

contains the cell wall antigen, there is evidence suggesting that the same epitope is also part of the antigen from the plasma membrane. First, the purified tetrahexose inhibits agglutination of latex-10G beads caused by both membrane extracts (Fr. III and IV) and cell wall extracts (Fr. I and II). Second, the immunogold-labeling on the plasma membrane and the cell surface are blocked when sections are incubated with the Mab 10G in the presence of the purified epitope. An alternative explanation is that the membrane antigen is structurally different but cross-reacts with Mab 10G. This seems unlikely, however, because monoclonal antibodies have been shown to discriminate microheterogeneity of antigenic determinants in yeast mannans (147).

Studies on *C. albicans* mannan structure and its carbohydrate determinants have provided the basis for serodiagnosis of medically important *Candida* species (95,346). Funayama et al (96) showed that *C. albicans* serotype A strains possess so called antigenic factors 1, 4, 5, and 6, while serotype B strains express antigenic factors 1, 4, 5, and 13b. The structural information of antigenic factor 6, which contains the serotype A-specific determinant, has been investigated by Suzuki et al (172,303,304) and Fukazawa and coworkers (94,96,326). Both groups have shown that factor 6 contains terminal  $\beta$ -1,2-linkages in the side chains as a factor-specific determinant (172,236). These terminal  $\beta$ -1,2-linkages in the mannan side chains are

resistant to mild acid treatment (304).

Monoclonal antibodies reactive with  $\beta$ -1,2-linked oligomannosyl residues of the phospho-mannan complex have been reported by others (336). However, these authors did not isolate the specific epitope to which the monoclonal antibodies recognized. In that report, the Mab specificities were determined by their reactivity with mannoproteins which contained the phosphate-bound  $\beta$ -1,2-linkages, and later screened for the negative reaction with mannoprotein fractions in which the phosphate-bound  $\beta$ -1,2-linked oligomannose had been hydrolyzed. Most of these Mabs had the same degree of reactivity with *C. albicans* serotype A mannoprotein fractions in which the phospho-mannan had been removed by acid hydrolysis. In our results, the low antigenic activity of the acid-modified mannoprotein suggests that Mab 10G is highly specific for the  $\beta$ -1,2-linked mannotetraose which reside in the acid-labile region, with little cross-reactivity with the  $\beta$ -1,2-linked mannosyl residues in the non-reducing terminal of antigenic factor 6 in serotype A strains (302,336).

Structural determinants other than factor 6 have also been reported. Shibata et al.(302) showed that the specificity of antigenic factor 5 lies within the phosphate-bound, acid-labile  $\beta$ -1,2-linked oligomannosyl residues. It appears that at least part of the epitope which is recognized by Mab 10G is also part of the antigenic factor 5

determinant. It is likely that Mab 10G has the potential to be utilized as a serodiagnostic agent for the identification of antigenic factor 5. Isolation of other mannosyl fragments from the cell wall mannans, and their reaction patterns with Mab 10G will add more information to the specificity, and possibly reveal the complete epitope to which the monoclonal antibody is specific.

Our results substantiate previous reports (146,335) in that *C. stellatoidea* and *C. tropicalis* contain  $\beta$ -1,2-linked mannosyl residues because Mab 10G agglutinated both species (Chapter 2). Since *C. stellatoidea* does not contain factor 6 determinant (169,335), this agglutination was probably caused by the phosphate-bound mannoses. *C. lusitaniae* and *C. intermedia* also contain the antigen recognized by Mab 10G (Chapter 2). The fact that *S. cerevisiae* was not agglutinated with Mab 10G does not exclude the possible existence of such residues in *S. cerevisiae*. It has been reported that *S. cerevisiae* has shorter phosphate-bound side chains than *C. albicans* (12,145). The significance in the different agglutination character between *C. albicans* and *S. cerevisiae*, and the possible functional role of the epitope during pathogenesis of candidiasis will be considered in Chapter 4.

## Chapter 4

### A DETERMINANT ON *C. ALBICANS* IS A SITE FOR ADHERENCE TO MOUSE SPLEEN MARGINAL ZONE MACROPHAGES

#### Introduction

The ability of *C. albicans* to adhere to host tissues is an important trait for tissue colonization and development of candidiasis (154,162,189,210,298). Studies have been done on mechanisms by which *C. albicans* attaches to epithelial cells (buccal, vaginal, and intestinal) (105,111,155,160-162, 165,314), extracellular matrix glycoproteins (166,212), fibroblasts (231), and inanimate surfaces (227,342). Factors, such as growth conditions, that influence *C. albicans* attachment have been reported (104,156,157). Alteration in cell surface features including hydrophobicity (121), topographical changes (228,343), and morphogenesis (161) have been implicated to affect adherence ability. However, the mechanisms and molecules responsible for dissemination of *C. albicans* yeast cells to organs and tissues as a result of candidemia are undefined.

Cell surface mannoproteins of *C. albicans* are strongly implicated as the adhesins responsible for adherence of the fungus to host cells (50,70,77,213,236,293,296,342). One

group of investigators reported that a carbohydrate determinant of a cell wall mannoprotein is involved in adherence of *C. albicans* to buccal epithelial cells (BECs) (236). Conversely, Tosh and Douglas (340) recently suggested that the protein portion is responsible for adherence of yeast cells to BECs. Reasons for this discrepancy are not clear, however, different adherence assays were used in these studies (viable colony counting versus direct cell counting). In addition, the *C. albicans* yeast cells were grown at different temperatures (37°C and 27°C). Lower temperatures have been shown to enhance surface hydrophobicity of *C. albicans* (118), cause non-specific adherence to mouse tissues (121), and the hydrophobicity appears to be due to the protein portion of cell wall mannoproteins (125). Alternatively, adherence of *C. albicans* to host cells may involve several adhesion systems and several kinds of molecules. In either case, the adherence of *C. albicans* to epithelial cells may not be applicable to systemic infections that result from yeasts in the circulation, such as that caused by intravascular catheters (3). Clearly, more knowledge on the interactions of *C. albicans* and internal organs is required to understand the mechanisms by which the fungus seeds tissues during the establishment of disseminated candidiasis.

The adherence specificity of *C. albicans* yeast cells for spleen marginal zone macrophages is described previously by

this laboratory (56,150). It was also demonstrated that 2-mercaptoethanol extract (Fraction I, Chapter 3) of whole yeast cells inhibits this adherence (Kanbe et al, manuscript submitted). In this chapter, I show that a specific cell surface antigen, which was recognized by a monoclonal antibody previously described (198), is one of the adhesin molecules responsible for the adherence of *C. albicans* to mouse spleen marginal zone. Furthermore, the antigenic determinant (epitope) recognized by the monoclonal antibody is the adhesin site on the polysaccharide antigen. The possible role of the determinant in the pathogenesis of candidiasis is discussed.

### Materials and Methods

#### Organisms and Culture Conditions.

*C. albicans* A9 (150) was obtained originally from an AIDS patient and was used for the adherence assay. Stock cultures were maintained in 50% glycerol in  $-20^{\circ}\text{C}$ . Yeast cells were grown at  $37^{\circ}\text{C}$  in GYEP broth under conditions described previously (Chapter 2). For adherence assay, three drops of 20 h-old culture suspension was inoculated into 25 ml fresh GYEP medium and incubated under the same conditions. Cultures were transferred three to four times before used for the assay. The stationary phase yeasts were harvested by centrifugation, washed three times in cold deionized water, and the cell number is determined by direct counting in a

hemacytometer. The hydrophobicity/hydrophilicity of the yeast cells was determined as described elsewhere (121). Yeast cells were used only if over 95% of the cells were hydrophilic. Washed yeast cells were centrifuged and the pellet was stored on ice and used within 2 h.

#### Immunoprecipitation.

The precipitation of antigen-antibody complex was first performed in a capillary tube to determine the optimal proportion of crude antigen extract (Zymolyase extract) to Mab 10G ascites (for Zymolyase extraction and ascites production, see Materials and Methods in Chapter 3 and 2, respectively). The reaction was then scaled up by adding 25 ml of fraction II at 30 mg/ml (750 mg) in PBS to 25 ml Mab 10G ascites (titer = 2,400, Chapter 2). The mixture was kept at 4°C for 48 h. The resulting precipitant was centrifuged and gently washed three times in 0.05M Tris-HCl buffer at pH 7.8 supplemented with 5 mM calcium chloride ( $\text{CaCl}_2$ , Sigma) and suspended in the same buffer. A non-specific *Streptomyces* protease (Pronase, Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to the suspension at a final concentration of 1 unit/ml. Sodium azide ( $\text{NaN}_3$ , Sigma) was also added to the suspension at a final concentration of 0.02%. The suspension was vigorously shaken after addition of Pronase and then incubated at 37°C. At time 12 h, 24 h, and 36 h, more Pronase (1 unit/ml) was added

to the suspension. At 48 h incubation, the reaction was stopped by boiling in a water bath for 15 min. The suspension was centrifuged and the remaining supernatant was exhaustively dialyzed against deionized water and freeze-dried.

#### Epitope Isolation.

The isolation and chemical characterization of the epitope recognized by Mab 10G was described in Chapter 3.

#### Adsorption of 2-Mercaptoethanol Extract with Latex-10G.

The procedure for solubilization of *C. albicans* adhesion molecules by 2-mercaptoethanol (2-ME) extraction was described in Chapter 3. To demonstrate that the antigen recognized by Mab 10G is an adhesin molecule in the 2-ME extract, the extract was absorbed with latex-10G before applied to the spleen sections (see below). One ml of 2-ME extract in Dulbecco's modified Eagle's medium (pH 7.4, Sigma) with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) supplemented with 5% BSA (Complete DMEM, CDMEM) was mixed with pelleted latex-10G beads (200  $\mu$ l of 1% solid suspension) or with the same amount of latex-BSA as a control for specificity. The suspension was kept on ice for 30 min, centrifuged, and the supernatant was adsorbed with another 200  $\mu$ l of either latex bead pellet. The adsorption was repeated four times. The agglutination of latex-10G by the extract during each adsorption was monitored. By the second

adsorption, no agglutination of latex-10G beads occurred when absorbed 2-ME extract was mixed with fresh latex-10G. The latex-adsorbed extract was prepared within 24 h before testing in the adherence assay.

#### Adherence Assay.

The procedures for *ex vivo* adherence assay were those described previously (281). Briefly, spleens were obtained from BALB/cByJ mice which have received intravenous (i.v.) injection of 0.1 ml 2% luconyl blue (BASF Aktiengesellschaft) 5 min earlier. Spleens were immediately placed in Tissue Tek O.C.T. compound (Miles Inc., Elkhart, Ind.), rapidly frozen on dry ice and stored at  $-80^{\circ}\text{C}$ . Ten  $\mu\text{m}$ -thick cryosections were cut at  $-20^{\circ}\text{C}$  (Frigocut 2800 N cryostat; Reichert-Jung, Leica, Inc., Deerfield, IL) and mounted onto Gold Seal Rite-On Micro Slides (Becton-Dickson Labware, Lincoln Park, NJ). The sections were air-dried and used for adherence assays within 1 h.

Sections on the slides were pretreated with 100  $\mu\text{l}$  of various concentration of different samples (see below) in CDMEM for 15 min at  $5^{\circ}\text{C}$  with 5 of the 15 min under rotation (Titer Plate Shaker, Lab-Line Instruments, Inc., Melrose Park, ILL). If samples contained solubilized cell wall adhesin, the adhesin should bind to ligand molecules on the host tissue, and block binding of yeast cells added subsequently (Kanbe et al, manuscript submitted). The

pretreatment fluid was decant, and 100  $\mu$ l of yeast cell suspension ( $1.5 \times 10^8$  yeasts/ml CDMEM) was added onto each section and incubated for 15 min without rotation at 5°C. The sections were fixed with 1.5% glutaraldehyde for 2 h at 4°C, washed in water, Gram-stained and coverslip mounted in Permount (281). Yeast cells which adhered to the marginal zone were counted by use of an image analyzer as described elsewhere (281). Cell numbers from five fields in one section were collected and averaged. Twelve sections were routinely counted for each treatment. Data are presented as average adherent yeast cells per field. Student t test was used for the statistical analysis.

The test samples used to pretreat splenic tissue sections included immunoprecipitated 10G antigen, 10G epitope, peak III from P-2 column (trimannose, see Chapter 3), 2-ME extract of whole yeast cells (Kanbe et al, manuscript submitted), 2-ME extract pre-adsorbed with latex-10G or latex-BSA beads, tetrahexose and trihexose of known structures (stachyose, maltotetraose, and raffinose; Sigma) as the negative control group. Stachyose and maltotetraose have the same molecular weight as that of the epitope. Raffinose is a trihexose and has the same molecular weight as that of peak III material. Control groups also included CDMEM only as a positive control for yeast cell tissue binding.

### Results and Discussion

As shown in Figure 37, pretreatment of spleen tissues with immunoprecipitated 10G antigen or the purified epitope inhibits the attachment of *C. albicans* to the marginal zone. The inhibition of *C. albicans* adherence by both the antigen and the epitope is dose-dependent. A trimannose fragment obtained from the P-2 chromatography of acid degraded mannoproteins also inhibited the adhesion of *C. albicans* to the spleen tissue (Figure 38), although not as potent as compared to the epitope. This mannosyl residue also contains 1,2-linkages and may have the same configuration as that of the epitope (Chapter 3). Inhibition of *C. albicans* attachment was not observed with other oligosaccharides even at the concentration of 1 mg/ml (Figure 39). These data suggest that the inhibitory activity of the antigen and the epitope is a result of specific interactions between the spleen tissue and the 1,2-linked mannosyl residues.

To determine whether the antigen recognized by Mab 10G is responsible for the inhibitory activity of the 2-ME extract, the extract was adsorbed with latex-10G before applying to spleen sections. Adsorption of the extract (5  $\mu$ g/ml) with latex-10G partially restored the attachment of *C. albicans* to the marginal zone, but there was still significant difference between the CDMEM control group and the latex-10G-adsorbed 2-ME extract group (Figure 40),

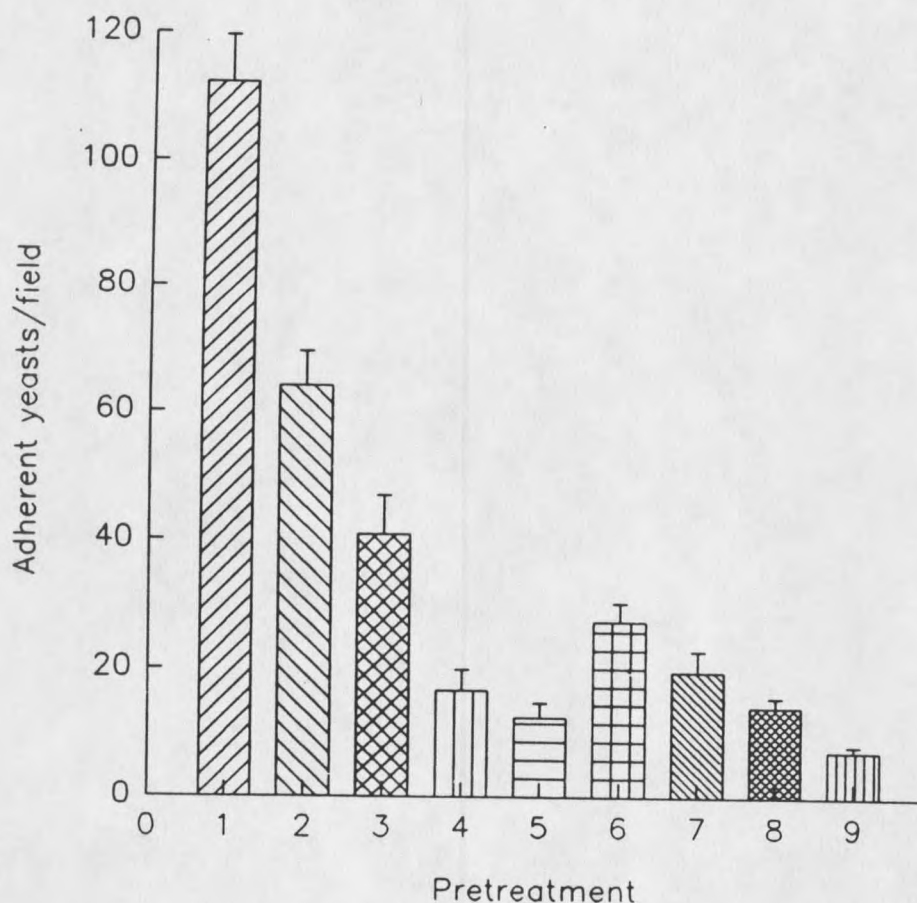


**Figure 37.** Inhibition of *C. albicans* adherence to mouse splenic tissue sections by immunoprecipitated 10G antigen (10G-Ag) and purified epitope. Spleen sections were pretreated with 100  $\mu$ l of various samples for 15 min at 5°C. (1) Medium (CDMEM) only. (2-5) 2, 10, 50, and 250  $\mu$ g (dry weight) of 10G-Ag/ml medium. (6-9) 2, 10, 50, and 250  $\mu$ g (sugar concentration determined by phenol- $H_2SO_4$  assay) of epitope/ml medium. Pretreatment fluid was decanted, and sections were then overlaid with 100  $\mu$ l of *C. albicans* yeast cells ( $1.5 \times 10^8$  yeasts/ml). After fixation and staining, the yeast cells that adhere to the marginal zone were counted. Data presented as adherent yeast/field. Error bars, standard error.

suggesting that there is more than one kind of adhesin in the 2-ME extract. A slight increase in yeast attachment may also be caused by non-specific adsorption of the beads (latex-BSA

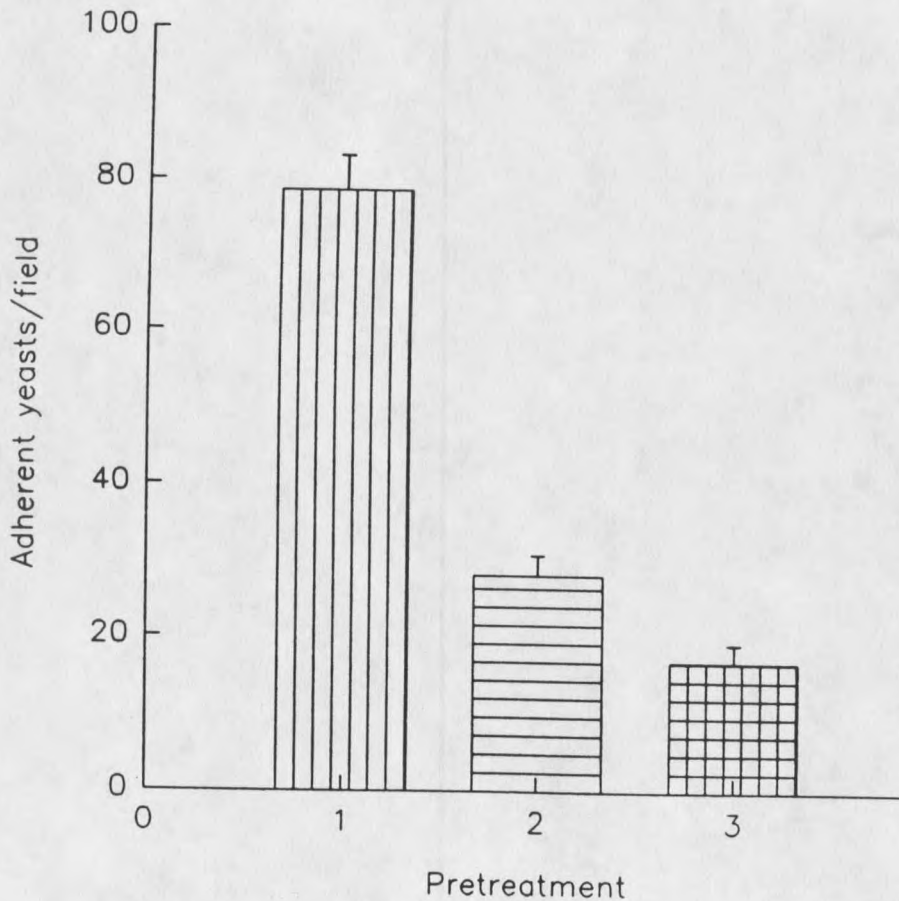


**Figure 38.** Inhibition of *C. albicans* adherence to mouse splenic tissue sections by a 1,2-linked trimannose (peak III). The trimannose was obtained as a smaller fragment as compared to the tetramannosyl epitope eluted from a size exclusion column (Li & Cutler, manuscript submitted). Spleen sections were treated with either CDMEM (1), 50  $\mu$ g peak III/ml medium (2), or 250  $\mu$ g peak III/ml medium (3) for 15 min. Sections were then treated under conditions described in FIG. 1. Error bar, standard error.

adsorption, Figure 40). At a 2-ME extract concentration of 10  $\mu$ g/ml, latex-10G adsorption did not significantly change the inhibitory effect of the extract, although the average binding did increase slightly. It is unlikely that the inability of latex-adsorption to remove the inhibitory

factors is due to incomplete adsorption of the antigen from the preparation. Because in both of the 2-ME extract concentrations used (5 and 10  $\mu\text{g/ml}$ ), the extracts were adsorbed twice more after they no longer agglutinate latex-10G. It is possible that molecules other than antigen 10G in the 2-ME extract are responsible for the post-adsorption inhibitory activities.

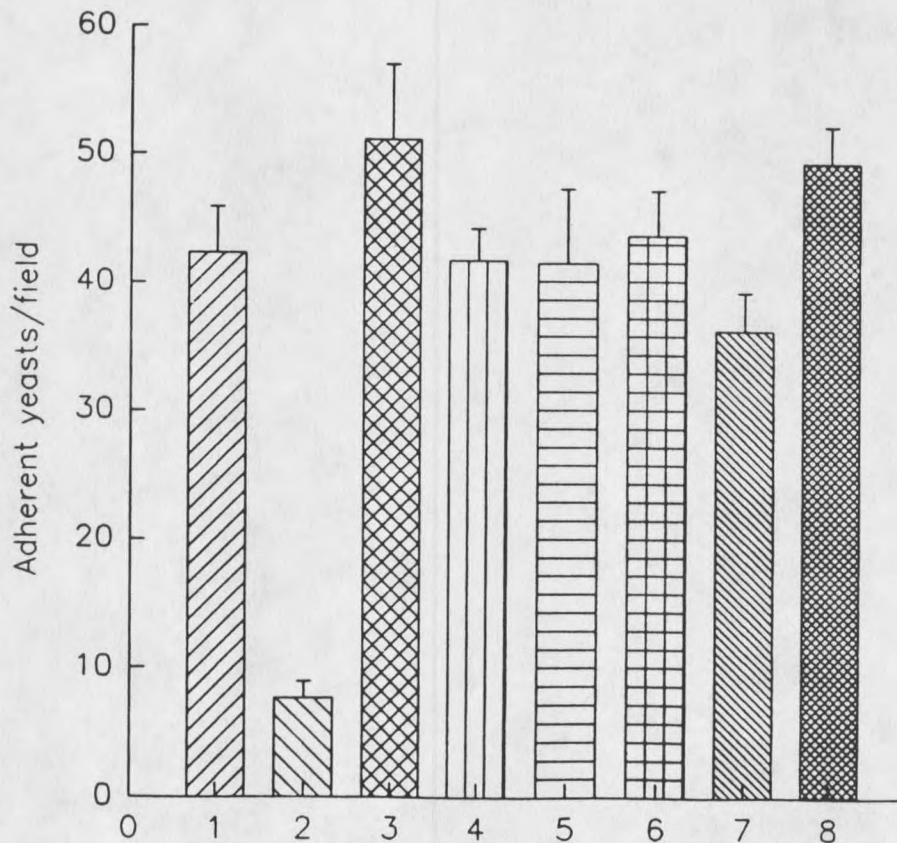


**Figure 39.** Effect of various sugars on the adherence of *C. albicans* to mouse splenic tissue sections. Assay methods are described in FIG. 1. Pretreatment solution include CDMEM (1), 5  $\mu\text{g}$  2-ME extract/ml medium (2), raffinose 250  $\mu\text{g/ml}$  medium (3), raffinose 1 mg/ml medium (4), maltotetraose 250  $\mu\text{g/ml}$  medium (5), maltotetraose 1 mg/ml medium (6), stachyose 250  $\mu\text{g/ml}$  medium (7), and stachyose 1 mg/ml medium (8). Error bars, standard error.

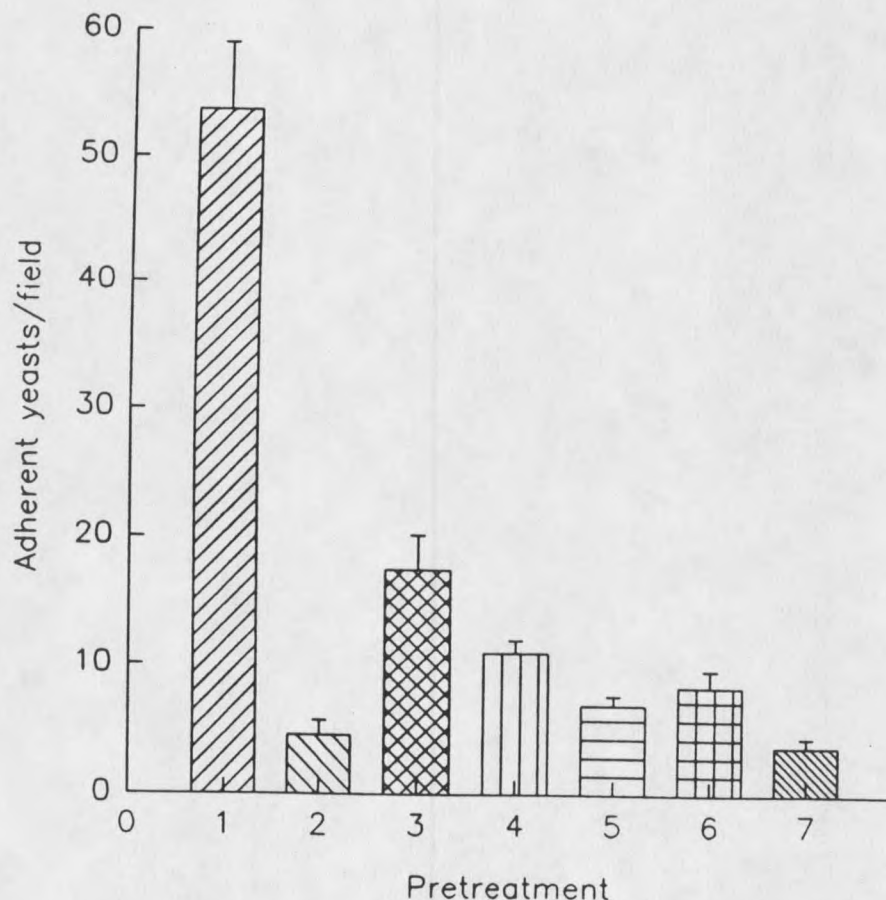


**Figure 40.** Latex-10G adsorption of 2-ME extract in the *ex vivo* adherence assay. 2-ME extract was mixed with latex beads conjugated with mAb 10G (latex-10G) and incubated at 4°C for 30 min. The beads were pelleted and fresh beads were added to remove any remaining 10G antigen from the 2-ME extract. The adsorption procedure was repeated four times. In the control group, 2-ME extract was adsorbed with latex beads conjugated with BSA (latex-BSA). The adsorbed 2-ME extract was used for the pretreatment of spleen sections in the *ex vivo* binding assay as described in the text. Pretreatment included (1) CDMEM only. (2) 2-ME extract 5 µg/ml medium. (3) 2-ME extract 5 µg/ml adsorbed with latex-10G. (4) 2-ME extract 5 µg/ml adsorbed with latex-BSA. (5) 2-ME extract 10 µg/ml medium. (6) 2-ME extract 10 µg/ml adsorbed with latex-10G. (7) 2-ME extract adsorbed with latex-BSA. Error bars, standard error.

Evidence for more than one kind of adhesin molecule is also supported indirectly. On a dry weight basis, the 2-ME

extract is a more potent inhibitor than the immunoprecipitated antigen. Fifty percent inhibition of *C. albicans* adherence to spleen tissue can be achieved by using less than 1  $\mu\text{g/ml}$  of 2-ME extract (Kanbe *et al*, manuscript submitted), whereas between 10 and 50  $\mu\text{g/ml}$  of immunoprecipitated 10G antigen is required for 50% inhibition (Figure 37).

The observation (Young *et al*, manuscript submitted) that when *S. cerevisiae* is grown as a hydrophilic yeast, it binds to the spleen marginal zone in a pattern much like that of *C. albicans*. However, Mab 10G does not agglutinate *S. cerevisiae* (see Chapter 2) suggesting that other molecules are involved in the adhesion mechanism for *S. cerevisiae*. The speculation of species-specific adhesion mechanism may also be applied to strains within the species of *Candida*. It was reported that mannoprotein isolated from one strain fails to inhibit adhesion of a second strain in the BECs model (50,229). Thus, the interaction and recognition between tissue and the fungus might be specific enough to distinguish the structural variations of mannoproteins among different species and strains.

Although it is generally recognized that cell wall mannoproteins of *C. albicans* are the effector molecules for tissue adherence, it is not clear whether the protein or the carbohydrate portion is responsible for the adherence ability. In one report, pretreatment of crude mannoprotein

preparation with heat, dithiothreitol, or proteolytic enzymes either partially or completely destroys its ability to inhibit *C. albicans* binding to BECs, whereas pretreatment with sodium periodate or  $\alpha$ -mannosidase has little or no effect (50). These authors suggested that the protein portion of the mannoprotein on *C. albicans* mediates the binding to a fucose-containing lectin on the buccal epithelial cells (BECs) (340). However, other workers in this laboratory have shown that the inhibitory activity of 2-ME extract was not affected by heat or proteolytic enzyme treatment, but was destroyed after periodate degradation (Kanbe et al. manuscript submitted), suggesting that the molecules involved in the adhesion of *C. albicans* to mouse spleen marginal zone are carbohydrates. Results reported here indicate that at least one of the adhesion molecules contains  $\beta$ -1,2-linked tetramannosyl residues.

Other reports on carbohydrate moiety of the mannoprotein as the effector for adherence to BECs have also been described. Sandin et al (292,293) found that  $\alpha$ -D-mannopyranoside treatment of buccal cells reduced adherence of *C. albicans* to BECs. Miyakawa et al (236) reported that *C. albicans* serotype B strains and mutant strains of serotype A, which lack the antigenic factor 6 determinant, exhibited significantly reduced ability to adhere to human BECs, suggesting that the carbohydrate determinants of factor 6 are involved in the epithelial adhesion. These authors reported

that strains of *C. guilliermondii*, which lack antigenic factor 5 and 6, exhibited only 5% adherence, much lower than those with only factor 6 deficiency. It was suggested that antigenic factor 5 may be involved in the adhesive ability of factor 6-deficient strains (236). I have previously discussed in Chapter 3 that the 10G epitope shares the same structures as that proposed for antigenic factor 5, it is thus likely that the epitope also plays a role in the adhesion of *C. albicans* to epithelial cells.

Mannose-mediated recognition has been described in other systems (357). It was reported that mannan from *S. cerevisiae* inhibits the splenic entry of lymphocytes by acting on a mannan receptor on the splenic sinusoidal cells (357). However, the detail chemistry of the mannan preparation was not defined in the study. Stahl *et al.* (316) have shown that macrophages express a cell surface receptor which mediates endocytosis and pinocytosis of particles containing mannose (316). It is not clear whether the adhesion of *C. albicans* to the spleen marginal zone macrophage involve the recognition of surface mannose by the 170 kd macrophage mannose receptor (87,333). Preliminary evidence show that the marginal zone attachment of *C. albicans* may be mediated by a different receptor because of its specific binding pattern and the factor that the adherence was not blocked by mannose. It is possible that the recognition of the  $\beta$ -1,2-linked tetramannose by spleen

marginal zone macrophages represents a novel mannose receptor yet to be determined.

The significance of the adherence pattern of *C. albicans* to the spleen marginal zone is not clear. It could be a host defense mechanism that is involved in the clearance of *C. albicans* from the circulation during fungemia (150) or a means for *Candida* dissemination into organs. Clearly, more information on the adhesion molecules and its tissue receptors are required in order to determine the adherence mechanism and its significance during the pathogenesis of candidiasis. The demonstration of  $\beta$ -1,2-linked tetramannosyl residues as one of the adhesins may serve as a basis for further investigation into the mechanisms of dissemination of *C. albicans*.

## Chapter 5

### CONCLUSIONS

- 1) An epitope recognized by a monoclonal antibody was found on the cell surface of *C. albicans* and on its plasma membrane as demonstrated by immunoelectron microscopy of yeast cells fixed by glutaraldehyde or freeze-substitution methods.
- 2) The expression of this antigen is primarily on the plasma membrane of the exponential growth phase yeast cells, and on the cell wall surface of stationary phase yeast cells.
- 3) Expression of the antigen by germ tubes of *C. albicans* is growth phase-dependent. The antigen is located in the subsurface area of the cell wall during early development of the germ tube, and becomes gradually expressed on the cell surface as the germ tube grows. In addition, dense expression of the antigen is observed on growing hyphal tips.
- 4) The antigen is found on other species of *Candida*,

including *C. stellatoidea*, *C. tropicalis*, *C. lusitaniae*, and *C. intermedia*. The antigen is not universally expressed because it was not detected on other fungi such as *Candida utilis*, *C. krusei*, *Cryptococcus neoformans*, *Cr. albidus*, *Torulopsis glabrata*, *Rhodotorula* spp. and *Saccharomyces cerevisiae*.

- 5) Freeze-substitution fixation methods greatly improved the ultrastructural preservation of plasma membranes, vacuoles, nuclei, and other cytoplasmic organelles as compared to the traditional glutaraldehyde fixation. Traditional chemical fixation caused lysis of spheroplasts and spontaneous membrane vesicle formation. Freeze-substitution, on the other hand, gave excellent preservation of both intact spheroplasts and association of antigen with cellular structures.
- 6) Tunicamycin, when added early in the growth phase of *C. albicans*, inhibits the antigen expression on the plasma membrane. Instead, the antigen accumulates in vesicle-like structures in the cytoplasm, indicating blockage by tunicamycin of the glycosylation during intracellular transportation of cell wall precursors across the plasma membrane.
- 7) Chemical analysis shows that the purified epitope, to

which the monoclonal antibody is specific, contains  $\beta$ -1,2-linked tetramannosyl residues. This epitope shares the same chemical structure as a portion of antigenic factor 5 of *C. albicans*. Both the epitope and factor 5 determinants are located in the phosphate-bound, acid-labile region on the oligomannosyl side chains of the cell wall mannan of *C. albicans*.

- 8) The isolated epitope and the immunopurified intact antigen block *C. albicans* adherence to mouse spleen tissues. The epitope was identified as one of the adhesion molecules involved in the binding of *C. albicans* to mouse spleen marginal zone macrophages.

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