



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

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

Montana State University

© Copyright by Diane Lynn Brawner (1985)

Abstract:

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

VARIABILITY IN SURFACE ANTIGEN EXPRESSION
OF CANDIDA ALBICANS

by

Diane Lynn Brawner

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

of

Doctor of Philosophy

in

Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

June 1985

D378
B739
cop. 2

ii

APPROVAL

of a thesis submitted by

Diane Lynn Brawner

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.

Date June 12, 1985 Jim E. Cutler by N.D. Reed
Chairperson, Graduate Committee

Approved for the Major Department

Date June 12, 1985 Norman R. Reed
Head, Major Department

Approved for the College of Graduate Studies

Date 13 June 1985 Michael P. Malone
Graduate Dean

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a doctoral degree at Montana State University, I agree that the Library shall make it available to borrowers under the rules of the Library. I further agree that the copying of this thesis is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this thesis should be referred to University Microfilms International, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute copies of the dissertation in and from microfilm and the right to reproduce and distribute by abstract in any format."

Signature Liane L. Browner
Date 6/12/85

ACKNOWLEDGMENTS

I would like to acknowledge and thank my major advisor, Dr. Jim E. Cutler, for his guidance, support, and encouragement throughout my project. I would also like to thank Dr. C.A. Speer, Sue Zaske, Dr. Larry Jackson, and Dr. Norman Reed for special advice and assistance.

TABLE OF CONTENTS

	Page
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
ABSTRACT.....	xi
INTRODUCTION.....	1
MATERIALS AND METHODS.....	20
Organisms and culture conditions.....	20
Serotyping of yeast strains.....	22
Immunization of mice	
Whole cell <u>Candida albicans</u>	
immunizations.....	23
Immunization with candidal ribosomal	
antigen fractions.....	24
Hybridoma production.....	24
Characterization of monoclonal antibodies	
Antibody class.....	25
Temperature stability.....	25
Precipitin tests.....	26
Purification of monoclonal antibody.....	26
Affinity chromatography.....	26
Ascites production.....	27
Production of normal mouse ascitic	
fluid.....	27
Production of ascitic fluid which	
contained monoclonal antibodies.....	27
Agglutination tests.....	28
Growth curve analyses	
Agglutination studies.....	29
Morphogenesis studies.....	29
Media comparison studies.....	30
Soluble crude antigen extraction and	
absorption.....	31
Indirect fluorescent antibody test.....	32
Immunospot assay.....	33
Indirect immunoelectron microscopy	
Location of surface antigens with	
ferritin labeling.....	35
Antigenic variability of yeast cells.....	35

TABLE OF CONTENTS (continued)

	Page
Preabsorption of diluted normal mouse ascitic fluid.....	37
Pretreatment of cells with fetal bovine serum and bovine serum albumin to to block nonspecific adherence of antibody.....	37
Trypsin treatment of germinating and nongerminating cells.....	38
Variability of surface antigens during morphogenesis.....	38
Enzyme-linked antigen distribution assay (ELADA).....	40
Background electron imaging (BEI) of colloidal gold particles.....	41
Characterization of antigens from crude antigen extract	
Heat stability.....	41
Antigen precipitation.....	42
Protein determinations.....	43
Carbohydrate analysis of crude antigen extract.....	43
Gel electrophoresis of crude antigen extract.....	43
Isolation of antigens from crude antigen extract	
Affinity chromatography.....	44
Protease treatment of antigen-monoclonal antibody precipitates.....	45
Characterization of isolated antigens	
Immunoelectrophoresis of antigens C6 and H9.....	50
Ultraviolet absorption spectra.....	50
High pressure liquid chromatography of AgC6 and AgH9.....	51
Lipid analysis.....	52
Carbohydrate analysis by gas-liquid chromatography and mass spectroscopy...	53
Hapten-inhibition tests.....	54
RESULTS.....	55
Serotyping of yeast strains.....	55
Production of monoclonal antibodies.....	55
Characterization of agglutinins.....	56
Purification of monoclonal antibodies.....	58

TABLE OF CONTENTS (continued)

	Page
Agglutination tests.....	58
Growth curve analyses.....	60
Effect of environment on agglutinin reactivity	64
Absorption and extraction procedures.....	66
FITC-labeled monoclonal antibody studies.....	69
Indirect transmission immunoelectron microscopy.....	70
Location of yeast phase surface determinants with ferritin-conjugated secondary antibody.....	70
Location of yeast phase determinants with colloidal gold-conjugated antibody.....	71
Preabsorption of diluted mouse ascitic fluid.....	76
Treatment to block nonspecific adherence of gold-conjugated antibody.....	76
Trypsin treatment of germinating and nongerminating cells.....	78
Variability of surface antigens during morphogenesis.....	81
Scanning electron microscopy	
Enzyme-linked antigen distribution assay (ELADA) to quantify surface antigen.....	85
Background electron imaging of <u>C.</u> <u>albicans</u> cells treated with gold conjugated antibody.....	85
Topographical surface changes of cells during germination.....	88
Characterization of antigens in crude antigen extract.....	92
Isolation of antigens from antigen extract..	95
Characterization of isolated antigens C6 and H9.....	97
DISCUSSION.....	128
LITERATURE CITED.....	148

LIST OF TABLES

Table	Page
1. Serotyping of yeast strains.....	56
2. H9 agglutinin reactivity with various yeast.....	59
3. Agglutination reactivity of yeast strains with monoclonal antibodies C6 and H9 at 24 h growth.....	61
4. Growth curve agglutination and TEM comparisons of the amount of surface antigen on <u>Candida</u> <u>albicans</u>	65
5. Effect of <u>C. albicans</u> nutritional environment on agglutinin H9 reactivity.....	67
6. H9 agglutinin absorptive ability of yeast strains.....	68
7. Detection of surface antigen of <u>C. albicans</u> by indirect immunoelectron microscopy.....	84
8. Evidence that protease treatment removes antibody from antigen-antibody complexes.....	96

LIST OF FIGURES

Figure	Page
1. Removal of antibody from ^3H antibody-antigen complexes by pronase digestion.....	48
2. Growth curve studies carried out on several yeast strains.....	63
3. Ferritin-labeled <u>C. albicans</u> cell.....	72
4. Labeling of <u>Candida</u> surface antigens with monoclonal antibody and colloidal gold-conjugated anti-immunoglobulin.....	73
5. Variable expression of gold-labeled antigens of surface features of <u>C. albicans</u>	77
6. Effects of trypsin treatment on specific and nonspecific binding of gold-conjugated antibody.....	79
7. Variable expression of antigenic determinants specific for monoclonal antibody H9 during morphogenesis of <u>C. albicans</u> 105.....	82
8. Variability in expression of the antigenic determinant for antibody C6 during morphogenesis of <u>C. albicans</u> 105.....	86
9. BEI photomicrographs of <u>C. albicans</u> 105.....	89
10. Germinating cells of <u>C. albicans</u> 105.....	90
11. TEM photomicrograph demonstrates collapse of cell wall.....	93
12. UV spectroscopy of a 5 mg/ml solution of crude antigen extract.....	98
13. UV spectroscopy of a 5 mg/ml solution of clarified AgC6.....	100
14. UV spectroscopy of a 5 mg/ml solution of clarified AgH9.....	101

LIST OF FIGURES (continued)

	Page
15. Ouchterlony double diffusion tests of HPLC-isolated AgC6 and AgH9 reacted with antibodies C6 and H9.....	102
16. Immuno-electrophoresis of HPLC-isolated antigens.....	103
17. Immuno-electrophoresis of HPLC-isolated antigens reacted with antibodies C6 and H9...	104
18. Spectrophotometric readings of fraction 1 and 2 of HPLC-isolated AgC6.....	105
19. Spectrophotometric readings of fraction 1 and 2 of HPLC-isolated AgH9.....	105
20. GLC elution profile of acetylated carbohydrate standards.....	107
21. GLC elution profile of acetylated AgC6.....	108
22. GLC elution profile of acetylated AgH9.....	109
23. GLC elution profile of acetylated carbohydrate standards on the DB-5 column....	111
24. Comparison of electron ionized fractionation patterns and retention times from acetylated carbohydrate standards.....	112
25. Elution profile of acetylated inositol standard with the DB-5 column.....	113
26. Electron ionized fractionation pattern of the inositol standard.....	114
27. Elution profile of acetylated AgC6 on the DB-5 column.....	115
28. Electron ionized fractionation patterns of acetylated AgC6.....	116
29. GLC elution profile of acetylated AgH9 on the DB-5 column.....	117
30. Electron ionized fractionation pattern of acetylated AgH9.....	118

LIST OF FIGURES (continued)

	Page
31. Fractionation patterns of acetylated inositol (standard) as determined by chemical ionization mass spectroscopy.....	121
32. Fractionation pattern of acetylated mannose standard as determined by chemical ionization mass spectroscopy.....	122
33. Fractionation pattern and molecular weight determination of acetylated AgC6 inositol (internal standard) component by chemical ionization mass spectroscopy.....	123
34. Fractionation pattern and molecular weight determination of acetylated AgC6 mannose component by chemical ionization mass spectroscopy.....	124
35. Fractionation pattern and molecular weight determination of acetylated AgH9 inositol component by chemical ionization mass spectroscopy.....	125
36. Fractionation pattern and molecular weight determination of acetylated AgH9 mannose component by chemical ionization mass spectroscopy.....	126

ABSTRACT

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

INTRODUCTION

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

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

draw direct parallels between other dimorphic fungi and C. albicans because C. albicans exists as a yeast at 25°C and germinates (M form) at 37°C. An argument flourishes over the basic question of whether the Y or the M form of C. albicans is responsible for candidiasis and attests to the impressive lack of knowledge concerning the organism.

Historically investigators associated parasitism with the M form and the saprobic state with the Y form (107).

However, others contest that this is an oversimplified view for several reasons. First, Y and M forms are usually found in tissue lesions (107); second, several reports indicate that only Y form cells are required for pathogenesis (129, 175); and third, other yeast (e.g. Torulopsis glabrata and Cryptococcus neoformans) are human pathogens but are incapable of M formation (134).

Theoretically, mycelial formation does endow the organism with certain advantages which may enhance pathogenicity:

1) the M form is resistant to phagocytosis (28, 32, 33, 81), and 2) hyphae confer a type of mobility which could enhance invasiveness and tissue penetration (107).

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

disseminated candidiasis do not present with pathognomonic symptoms and blood cultures are often negative.

Therefore, attempts have been made to diagnose disseminated disease by seroassay (for antibody specific for C. albicans). Unfortunately, the presence of C. albicans on mucosal surfaces of colonized (non-diseased) individuals promotes antibody production (7, 65).

Therefore, unless semi-quantitative determinations or serial titers of antibody are done, serodiagnosis is a meaningless indicator of infection. These techniques are also crude in that, presently, tests often rely on poorly defined and unstandardized antigen preparations which preclude reliability. Finally, tests to detect serum antibody may be falsely negative in immunosuppressed people, the population which presently is in greatest danger of disseminated candidiasis.

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

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

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

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

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

MATERIALS AND METHODS

Organisms and culture conditions.

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

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

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

Antigenic variability studies in morphogenesis.

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

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

Serotyping of yeast strains

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

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

Immunization of mice

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

Immunization with candidal ribosomal antigen

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

Hybridoma production

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

Agglutinin reactivity was detected directly by combining hybridoma culture supernatant material with heat-killed C. albicans 9938 blastoconidia. Hybridoma antibody production was determined indirectly by using an enzyme immunosorbent assay (BRL Hybridoma Screening Kit, Bethesda Research Laboratories, Gaithersville, MD). All antibody-producing hybrids were subcloned by limiting dilution a minimum of 3 times. Three of the hybrids, C6, B6, and H9, produced antibody that agglutinated C. albicans 9938. Nine additional nonagglutinating IgM (5), IgG₁ (1), and IgG₂ (3) monoclonal antibodies were also produced, but were not characterized beyond class designation or used in experiments.

Characterization of monoclonal antibodies

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

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

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

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

Purification of monoclonal antibody

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

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

Ascites production

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

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

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

Agglutination tests

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

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

Growth curve analyses

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

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

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

Media comparisons studies

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

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

Soluble crude antigen extraction and absorption

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

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

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

Indirect fluorescent antibody test

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

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

Immunodot-spot assay

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

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

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

Indirect immunoelectron microscopy

Location of surface antigen with ferritin labeling.

Yeast cells were grown in GYEP broth for 18 h at 37°C with rotation (160 rpm). Cells were washed in 0.01 M PBS, reacted with 1.0 ml of either H9 monoclonal antibody (agglutinin titer = 12,500) or absorbed (described above) normal ascitic fluid, washed in PBS, and reacted with 0.1 ml of undiluted goat anti-mouse ferritin-conjugated antibody (Cappel) for 30 min at 22-24°C with rotation (160 rpm). Yeast cells were washed in PBS, fixed (0.25% formalin), and embedded in 2% agarose which was subsequently cut into 1 mm cubes. Cubes were post-fixed in 4% OsO₄ for 12 h at 4°C, rinsed in cold water (pH 7.2-7.4), dehydrated through an ethanol series, embedded in Spurr's resin (147), thin sectioned, post-section stained with lead citrate and uranyl acetate, and examined in a Zeiss EM9S-2 electron microscope.

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

of C. parapsilosis, grown to stationary phase in GYEP broth for 24 h, were washed 3 times in sterile distilled water, and 5×10^7 organisms (by hemacytometer count) were inoculated into 200 ml of fresh GYEPB in a 500 ml Erlenmeyer flask. Cultures were incubated at 37°C with rotation (160 rpm). At 4h, 6h, 10h, 24h, and 48h, aliquots of medium containing 10^8 cells were removed, washed 3 times in ice-cold 0.01M PBS pH 7.2, fixed in 0.25% formalin for 15 min at 4°C, reacted with 0.5 ml of mouse ascitic fluid containing monoclonal antibody H9 or C6 (agglutinin titer = 6400) or with normal mouse ascitic fluid (negative control) for 30 min at 23 to 25°C with rotation (200 rpm), and washed 3 times in 0.01M PBS. Goat anti-mouse antibody conjugated with 20 nm colloidal gold particles (40 ug/ml protein, E-Y Laboratories, Inc., San Mateo, CA) was diluted 1:2 in 0.01M PBS, and 0.1 ml was added to the yeast for 90 min at 23 to 25°C with rotation (200 rpm). Cells were washed 4 times in 0.01M PBS, prefixed in 0.5 ml of 0.25% formalin, fixed (0.5% glutaraldehyde, 1.0% acrolein, and 0.2 M sucrose in 0.075M PBS pH7.2 for 30 min at 23 to 25°C); post-fixed for 1 h in 1% OsO₄, dehydrated through a series of graded ethanol, embedded in Spurr's low viscosity resin (147), and thin sectioned. Sections were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (TEM) in a JEOL-100 CX electron microscope. A

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

Preabsorption of diluted normal mouse ascitic fluid.

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

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

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

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

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

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

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

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

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

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

Characterization of antigens from crude antigen extract

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

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

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

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

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

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

Carbohydrate analysis of crude antigen extract. The colorimetric method of Dubois et.al. (36, H₂SO₄-phenol method) was used to determine the carbohydrate concentration in crude antigen extracts.

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

37.5 to 150 ug in 40 ul of Laemmli loading buffer. The gels were run at constant voltage (120 V). High molecular weight standard proteins (Sigma) were run as controls in protein gels. Duplicate PAGE gels were run simultaneously to detect carbohydrate. Lanes were loaded with samples ranging from 30 to 120 ug carbohydrate in 40 ul loading buffer. Fetuin (20 ug, Sigma), mannose (12.5 ug, Sigma), and dextran (12.5 ug, Pharmacia) were run as controls. Protein gels were fixed in 5% TCA for 30 min and stained for 2 h in 0.01% Coomassie brilliant blue (Sigma). Carbohydrate gels were stained with Schiff's Reagent (82).

IEF flat-bed discontinuous polyacrylimide (10%) gels were also run on the antigen extract in 0.075 M barbitol buffer (Gelman, Ann Arbor, MI) under non-reducing conditions at 100 V for 2 h.

Isolation of antigens from crude antigen extracts

Affinity chromatography. CNBr activated 4B Sepharose columns were prepared as described by Cutler and Poor (28). Briefly, 4B Sepharose was washed in distilled water, activated with finely-ground CNBr (1.0 g/10 ml beads) at pH 11 and 20°C, cooled to 4°C, vacuum filtered, and washed with 0.01 M borate buffered saline pH 9. Beads were packed by centrifugation (13xg for 10 min) and 2.5 ml of monoclonal antibody C6 or H9 (titer = 12,800) in

ascitic fluid or normal mouse ascitic fluid was added to 2.5 ml beads and incubated at 4°C for 12 h with rotation (50 rpm). The supernatant fluid from the beads was tested for a decrease in titer of monoclonal antibody by tube agglutination. Beads were washed in PBS and packed into 5 x 1.5 cm columns. Fifty ml of PBS were run through each column for equilibration. One ml of a 5 mg/ml solution of crude antigen extract was added to the top of each column and chased with 15 ml of PBS. Fifteen ml of eluate were collected from each column and concentrated by negative pressure dialysis (28). Protein determinations were made on the eluate fluid (using 260/280 comparisons as described above) and concentrated eluant fluid was tested by Ouchterlony double diffusion for evidence of unattached antigen. Elution of antigen from antigen-antibody complexes which had formed was attempted either by addition of 0.5 M mannose and 0.5 M glucose, by low pH (glycine buffer pH 2), or by high salt concentration (1-4 M NaCl).

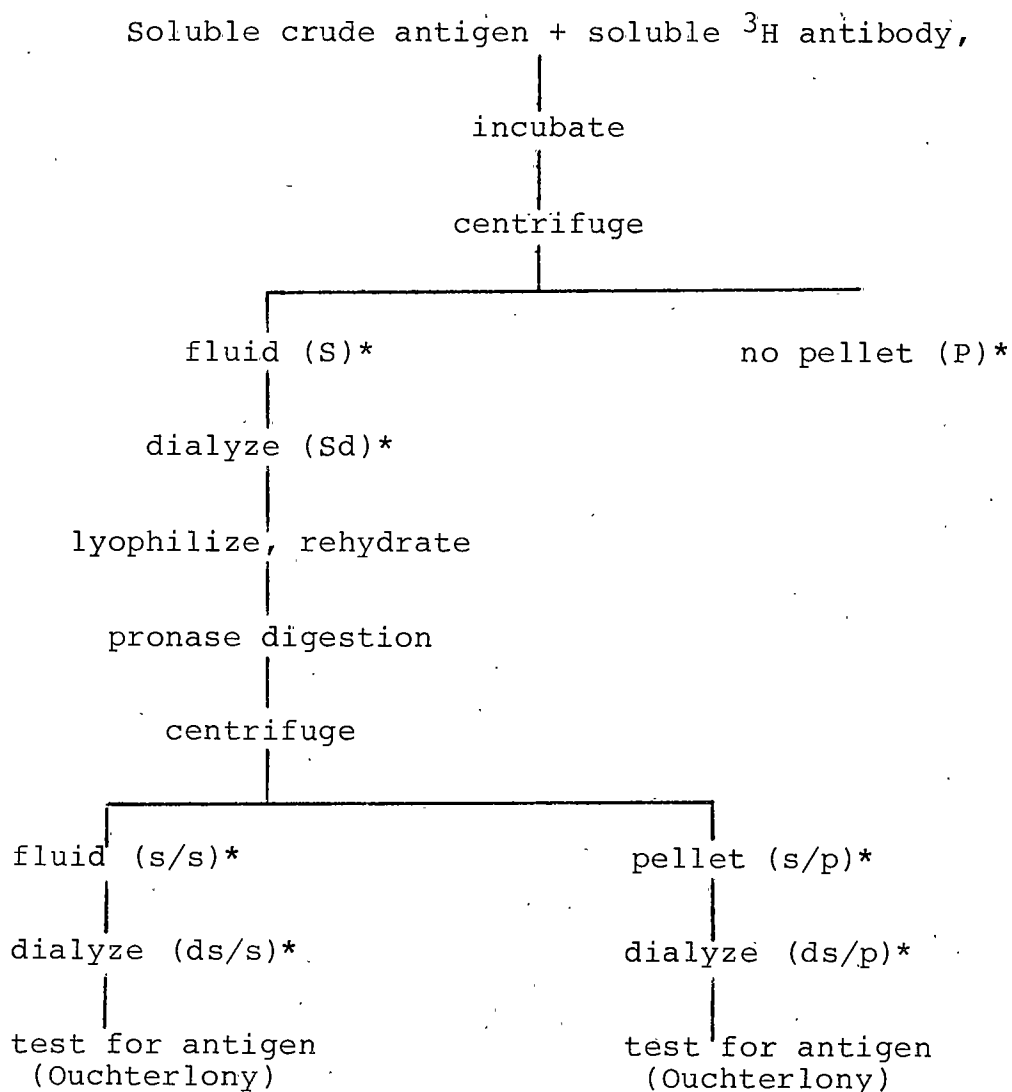
Protease treatment of antigen-monoclonal antibody precipitates. Qualitative capillary tube precipitation tests (43) were done to determine the optimal proportions of crude antigen extract and monoclonal antibody (in pooled mouse ascitic fluid) for both C6 and H9 monoclonal antibodies. Crude antigen extract in concentrations varying from 5-105 mg/ml was solubilized in saline and

added to pooled, agglutinin-titered (25,000 and 6400 for H9 and C6, respectively) monoclonal antibody in ascitic fluid. The time of precipitin formation was recorded and the mixture which produced the most rapid precipitate was considered to represent the optimal antigen/antibody proportions. I then scaled up the reaction to harvest larger quantities of precipitate by adding 75 ml of titered H9 antibody to 750 mg of crude antigen or 56 ml of titered C6 antibody in ascitic fluid to 494 mg of crude antigen extract. Mixtures were refrigerated for 48-72 h to allow precipitate formation, centrifuged (15,000xg) for 20 min and the antigen/antibody precipitates were gently washed in saline and resuspended in Tris-CaCl₂ buffer pH 8.2 (TCB, 0.05 M Tris-HCl/ .005M CaCl₂). A solution of Pronase B (Streptomyces protease, 4 mg/ml Calbiochem-Behring Corp.), was made in TCB, allowed to self-activate at 37°C for 15 min, and aliquots were frozen at -20°C. Protease treatment of the antibody-antigen complex was done [modification of methods described by Brenkle and Kornfeld (12)]. At 0, 12, 24, 36 h, and 48 h, 25 ul of the 4 mg/ml protease solution was added to the antigen-antibody precipitate at 37°C under toluene. The mixture was vigorously agitated after each addition of enzyme. At 60 h, the protease was inactivated in a boiling water bath for 15 min, centrifuged (1000xg), and supernatant fluid was exhaustively dialyzed against distilled water, tested

for antigen reactivity by Ouchterlony double diffusion against C6 and H9 monoclonal antibodies (titer of each = 6400), and lyophilized.

Figure 1 represents a summary of methods used to determine if protease treatment of antigen-antibody precipitates was effective in removing monoclonal antibody from the antigen-antibody complexes. Hybridoma cell lines C6 and H9 were grown in RPMI 1640 + 10% FBS for 3 d. Modified RPMI 1640 which contained 10% of 15 L-amino acids including alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine and 10% FBS was made. Cells were washed once, counted (hemocytometer) and 2×10^5 cells were resuspended in 0.9 ml of the modified medium. Tritiated amino acid reagent (0.1 ml, 1 mCi/ml, Schwartz-Mann, Cambridge, MA) was added and cells were incubated for 20 h at 37°C in 5% CO₂. Cell supernatant fluids (50 ul) containing ³H-labeled monoclonal antibodies C6 or H9 were reacted with 10^4 whole C. albicans 9938 or S. cerevisiae cells which had first been treated with 10% fetal bovine serum in saline to block nonspecific binding of the antibody. Cells were washed twice in PBS and counted 3 times in a Packard Tri-carb 460 CD Liquid Scintillation Counter (Downers Grove, IL) to ensure the presence of ³H-labeled specific antibody. C. albicans 9938 bound over seven

Figure 1. Removal of antibody from ^3H antibody-antigen complexes by protease digestion.



*Scintillation counts done at these points.

times more antibody than S. cerevisiae cells (747 cpm as compared to 97 cpm, respectively). Labeled antibody (1.0 ml) was then reacted with 2.0 mg of crude antigen extract and incubated at 22-24°C for 2 h with rotation (5xg), centrifuged (11,000 rpm in a Eppendorf centrifuge for 15 min), and the supernatant fluid was dialyzed exhaustively against normal saline and lyophilized. The lyophilized material (containing antigen-antibody complexes) was resolubilized in 1.0 ml in Tris-CaCl₂ buffer, protease-treated as above at 0, 12, 24, 36, and 48 h, heat-inactivated at 60 h, and dialyzed exhaustively against saline. Crude antigen extract in unlabeled amino-acid medium (without antibody), unlabeled monoclonal antibody in amino acid-free medium (without antigen extract), and 10 ul ³H-labeled amino acid reagent in amino acid-free medium were also subjected to the enzyme treatment. Scintillation counts were done [10 ul of material in 3.0 ml Optisol (Isolab, Akron, OH)] in triplicate at each step for 5 min (Packard) as indicated in Figure 1. Dialysate fluid of fractions was monitored by scintillation counting and dialysis was continued until counts (of the dialysate fluid) were reduced to background levels before proceeding with the experiment. Background counts were established by including the following controls (all suspended in 3 ml Optisol): 10 ul of Tris-CaCl₂, 10 ul of amino acid-free medium, 10 ul solubilized crude antigen extract

(both supernatant fluid and pellet, see Figure 1), unlabeled monoclonal antibodies (both supernatant fluid and pellet), and these counts were compared to counts obtained when Optisol alone was counted. An unquenched ^3H standard (Packard), and 10 μl of ^3H -labeled amino acid reagent in 3 ml Optisol were also counted to monitor counting efficiency and establish maximum attainable cpms. Ouchterlony double diffusion tests were done to determine the fraction which contained the antigens throughout the experiment and to establish that the reactivity of the antigen was undamaged by protease treatment.

Characterization of isolated antigens

Immunoelectrophoresis of antigens C6 and H9. Isolated (protease-treated) AgC6 and AgH9 were electrophoresed in 1% agarose for 1 h and 3 h in Veronal buffer pH 8.6 at 100 V. Troughs were cut into the agarose and antibodies C6 and H9 were added and allowed to diffuse and react with electrophoresed antigens at 22-24°C for 72 h.

Ultraviolet absorption spectra. Five mg of antibody-precipitated, protease-treated antigens AgC6 and AgH9 were suspended in 1.0 ml distilled water, centrifuged (11,000 rpm for 2 min in an Eppendorf centrifuge), and optical density was determined in UV scans (range 190-340 nm, Gilford 250 Spectrophotometer). A scan was also done on

clarified (centrifuged) and unclarified 5 mg/ml solution of crude antigen extract.

High pressure liquid chromatography (HPLC) of AgC6 and AgH9. HPLC was done with a Beckman model 332 gradient liquid chromatograph equipped with model 110 A pumps and a BD 40/41 recorder with UV absorbance detector (254 nm). Two columns were used. The first was a 25 cm x 4.6 mm I.D. reverse phase column (Ultrasphere-ODS, Beckman Instruments, Inc, Fullerton, CA) and 1.0 ml of a 5 mg/ml suspension of either H9 or C6 Pronase B-purified (isolated) antigen was loaded onto the column in 250 ul aliquots. Flow rate was maintained at 1 ml [70% H₂O:30% (v/v) acetonitrile]/min. Two fractions of 15 ml each were collected, lyophilized, rehydrated (0.5 ml H₂O) and 18 ul were tested for activity by Ouchterlony double diffusion tests. Both fractions obtained from both antibodies were also run on a 7.5 mm ID x 30 cm sizing column (Spherogel TS-K 3000 SW, Altex, a division of Beckman) in H₂O at 1.2 ml/min. One ml fractions were collected and absorption of each fraction was determined on a Gilford 250 Spectrophotometer at 220 nm (Figures 18 and 19). Fractions which contained peaks (fractions 5-8, AgH9; and fractions 6-8, AgC6) were pooled and lyophilized in preweighed flasks. Flasks were weighed after lyophilization and residues rehydrated in 0.5 ml H₂O. All fractions which were not included in peaks (tubes 9-20 for

both antigens) were also pooled, lyophilized in preweighed containers and the weight of the lyophilized residue was determined by weighing containers after lyophilization. Eighteen microliters of each fraction were used to test for reactivity with antibodies C6 or H9 in Ouchterlony double diffusion tests. Ten microliters each of Blue dextran (2.0 mg/ml), phosphorylase b (25 mg/ml), bovine serum albumin (25 mg/ml) and cytochrome c (25 mg/ml) were used as controls to determine void volume and separation capability of the columns.

Lipid analysis. AgC6 and AgH9 (protease-treated) and crude antigen extract were tested for the presence of fatty acids. One mg each of antigen preparation, phosphatidyl choline, and araditic acid were hydrolysed in 2N trifluoroacetate anhydride (TFA) for 1 h at 121°C. TFA was evaporated off at 50°C under a stream of N₂, and residues of lipid extracts were rehydrated in distilled water. One microliter of hexane was added, and after mixing the hexane supernatant layer was removed and evaporated at 50°C under a N₂ stream. The hexane extraction procedure was repeated 3 times. Fatty acids were graciously analyzed by Dr. Larry Jackson with analytical thin-layer chromatography (TLC) on silica plates (Absorbosil 2, Applied Science Laboratories), and developed in hexane-diethyl ether-acetic acid (85:15:1, vol/vol).

Visualization was done by spraying with sulfuric acid-potassium dichromate followed by charring.

Carbohydrate analysis by gas-liquid chromatography (GLC) and mass spectroscopy. Neutral sugars (in isolated antigen preparations) were derivatized to alditol acetates and analyzed by GLC methods previously described (2). Fractions H9₅₋₈ (1.2 mg) and C6₆₋₈ (3 mg) each containing 1 mg meso-inositol (internal standard, General Biochemicals, Chagrin Falls, OH) were hydrolysed in 2N trifluoroacetate anhydride (Eastman Organics, Rochester, NY) for 1 h at 121°C, and reduced to alditols with NaBH₄ (Sigma, 10 mg/ml) in 1.0 N NH₄OH (Baker) for 1 h at 22-24°C. Borate was removed by 1.0 ml methanol evaporations (repeated 5 times), and alkylation was done by addition of 1.0 ml acetic anhydride (Sigma) in sealed tubes at 121°C overnight. Several known carbohydrate standards (glucose, galactose, mannose, N-acetylglucosamine, (Sigma) and meso-inositol (General Biochemicals, 1.0 mg each) were also derivatized as above. One microliter fractions of all samples were analyzed on a glass column (2m x 2mm ID) of 3% ECNSS-M on 100/120 mesh Gas-Chrom Q (Applied Science Laboratories) programmed from 190-210°C at 2°C/min. The gas carrier flow rate was 30 ml/min. Mass spectroscopy-GLC analysis was done with 50 nl samples of derivatized antigen preparations and standards (prepared as described above) on a Varian 3700 gas chromatograph equipped with a

DB5 capillary column (30 mm x 0.25 mm ID) which was interfaced directly to the NG 7070E-HF double-focusing high resolution mass spectrometer at an accelerating voltage of 6.0 kV, an electron energy of 70 eV, and a source temperature of 200°C. The initial temperature was held at 50°C for 3 min and ramp rate was 20°C/min to 180°C and 2°C/min to 230°C.

Hapten-inhibition tests. Modified Ouchterlony tests were done by testing monoclonal antibodies C6 and H9 with various concentrations of crude antigen extract in the presence of 10% carbohydrate. One percent agarose plates were incorporated with 10% (w/v) of one of the following: glucose, mannose, i-inositol, meso-inositol, galactose, arabinose trehalose, or rhamnose (Sigma). A plate which contained only 1% agarose served as a control. Wells were cut into the agarose in the pattern shown in Figure 15. The center well was loaded with 18 μ l of either C6 antibody (titer = 1600) or H9 antibody (titer = 3200). Wells 1-5 were loaded with 18 μ l of a 5, 2.5, 1.0, 0.5, or 0.1 mg/ml saline suspension of crude antigen extract. Plates were incubated 72 h at 22-24°C and examined for evidence of precipitin bands.

RESULTS

Serotyping of yeast strains

Eight strains of yeast, examined by growth curve analyses (see below), could be grouped according to H9 agglutinin reactivity. We questioned whether these groups corresponded to the serotypes of the 8 strains. The results of serotyping 8 strains of C. albicans and 1 strain each of C. tropicalis and T. glabrata are shown in Table 1. Three yeast strains, C. albicans 9938, 4, and 19 serotyped as group A and C. albicans 394, 17, and 34 serotyped as group B. Serotyping groups did not correspond with groups formed by reactivity with H9 antibody.

Production of monoclonal antibodies

The fusion of splenocyte and myeloma cells to produce hybridomas often results in genetically unstable cell lines. Therefore, although over 100 splenocyte-myeloma cell fusions were done and initially yielded over 300 antibody-producing clones, many of these positive hybrid cells lost vigor or antibody-producing capabilities during subsequent cloning and isolation procedures. As a result, 12 monoclonal antibodies survived all isolation and cloning procedures; 3 were agglutinating IgM class

antibodies (which are described here), 4 were IgG class antibodies (3 IgG₂, 1 IgG₁) and 5 were nonagglutinating IgM class antibodies. No hybrid antibody-producing clones survived in fusions which were produced from mice immunized with ribosomal fractions.

TABLE 1. Serotyping of yeast strains.

<u>Organisms</u>	<u>Serotype</u>	<u>Grouping by H9 reactivity</u>
<u>C. albicans</u> 9938	A	I
394	B	II
4	A	III
17	B	III
19	A	III
34	B	III
28367	A	
38696	B	
<u>C. tropicalis</u> 4	A	
<u>T. glabrata</u> 15	A	I

Characterization of Agglutinins

Enzyme-linked immunosorbent assays were positive for antibody production 3 weeks after splenocyte-plasmacytoma fusions and agglutinin activity of the

culture fluid was simultaneously observed. The 3 hybrid cell lines which produced agglutinating monoclonal antibodies B6, C6, and H9 were stable and produced antibody for at least 50 tissue culture passages. In Ouchterlony double diffusion tests a single precipitin band developed between anti-mouse IgM (heavy chain specific) and each agglutinin when hybridoma supernatant fluid was concentrated 5 fold, but were nonreactive with goat antibodies specific for other mouse immunoglobulins (Meloy Laboratories, Inc., Springfield, VA). In addition, a single precipitin band developed in immunoelectrophoretic analysis between each electrophoresed agglutinin and anti-whole mouse serum. This band was coincident with IgM migration when reacted with H9, C6, and B6. Single precipitin bands developed when B6, C6 or H9 were tested against antigen extracts in Ouchterlony double diffusion analyses, and nonidentity was noted in precipitin bands formed between C6 and H9, and B6 and H9. Bands of identity formed between C6 and B6 precipitin bands.

Heating agglutinin H9 to 60°C for 5 min destroyed its ability to agglutinate C. albicans 9938 cells, but agglutinins C6 and B6 were stable to heating at 56°C for 1 h. All 3 monoclonal antibodies retained activity after freezing at -20° and -70° and after lyophilization.

Purification of monoclonal antibodies

Attempts to attach antigen from crude antigen extract to Sepharose 6B with divinyl sulfone were unsuccessful. Tube agglutination titers were identical when done on the eluates from the divinyl sulfone activated, antigen-treated and untreated beads. Although antibody H9 and C6 in ascitic fluid (titer = 6400 and 8000, respectively) were successfully attached to Sepharose 4B with CNBr and bound antigens (as determined by Ouchterlony diffusion tests), attempts to utilize this method to purify antigens were unsuccessful in that antigens could not be eluted from the antigen by washing with 50 ml of 0.5 M mannose, 0.5 M glucose, high salt concentration (0.5 - 4.0 N NaCl), or glycine-HCl buffer pH 2.2.

Agglutination tests

The slide agglutination titer of pooled hybridoma culture fluid (C6 and H9) was 8 with C. albicans 9938. Concentration of the H9 agglutinin by 50% $(\text{NH}_4)_2\text{SO}_4$ fractionation increased the titer to 32 and produced a tube agglutination titer of 256.

Of the 96 strains of C. albicans tested for agglutinin H9 reactivity, 19 strains which were nonreactive with unconcentrated agglutinin were reactive when tested with the $(\text{NH}_4)_2\text{SO}_4$ -concentrated agglutinin (Table 2). All

Table 2. H9 agglutinin reactivity with various yeasts.

ORGANISM ^a	NUMBER OF STRAINS	AGGLUTINATION REACTION ^b
<u>CANDIDA ALBICANS</u>	42 ^d	3-4+
	15 ^e	2+
	19 ^f	1+
	19 ^g	- c
<u>C. ALBICANS VAR. STELLATOIDEA</u>	1	+
<u>C. TROPICALIS</u>	7	2-4+
<u>C. PARAPSILOSIS</u>	1	-
<u>C. KRUSEI</u>	1	-
<u>TORULOPSIS GLABRATA</u>	2	4+
	1	2+
<u>CRYPTOCOCCUS NEOFORMANS</u>	1	-
<u>C. TERREUS</u>	1	-
<u>RHODOTORULA SP.</u>	1	-
<u>SACCHAROMYCES CEREVISIAE</u>	1	-
<u>TRICHOSPORON BEIGELII</u>	1	-
<u>PHAEOCOCCOMYCES EXOPHILIAE</u>	1	-
<u>BLASTOSCHIZOMYCES PSEUDOTRICHOSPORON</u>	1	-

^a YEASTS WERE GROWN IN GLUCOSE-YEAST EXTRACT-PEPTONE BROTH AT 37°C FOR 24 H (48 H FOR LAST 3 ORGANISMS).

^b SCORING OF REACTIONS FROM NO EVIDENCE OF AGGLUTINATION (-) TO MAXIMUM AGGLUTINATION (4+)

^c POSITIVE AGGLUTINATION OCCURRED WITH ANTIBODY CONCENTRATED BY (NH₄)₂SO₄ FRACTIONATION.

^d INCLUDES C. ALBICANS 9938, C. ALBICANS 9; 81% WERE RECENT CLINICAL ISOLATES.

^e 67% WERE RECENT CLINICAL ISOLATES

^f INCLUDES C. ALBICANS 17, 34, AND 394; 79% WERE RECENT CLINICAL ISOLATES.

^g INCLUDES C. ALBICANS 19; 68% WERE RECENT CLINICAL ISOLATES.

strains of C. tropicalis and 3 strains of T. glabrata were reactive , but none of the other yeasts agglutinated with unconcentrated or concentrated agglutinin.

Agglutinin reactivity of 29 strains of C. albicans to C6, B6, and H9 agglutinins was compared. All strains reacted similarly with monoclonal antibodies C6 and B6 which suggested that these 2 antibodies are specific for the same epitope. Therefore, all further studies were confined to reactions involving C6 and H9. In 18 of 29 strains of C. albicans tested with C6 and H9 monoclonal antibodies, the reactivity was positive to the same degree. In 4 strains, H9 reacted more strongly and in 9 cases C6 reacted more strongly (Table 3). H9 reacted strongly with T. glabrata while C6 was nonreactive. With the exception of C. tropicalis, other species of Candida did not react with either antibody.

Growth curve analyses

Using agglutinin H9, we examined cell surface determinant expression as a function of growth in nine yeast strains. Six of these strains were chosen on the basis of their reactivity with the unconcentrated agglutinin: C. albicans 9938 and T. glabrata agglutinated strongly while C. albicans 394, C. albicans 17, C. albicans 19 and C. albicans 34 were weakly reactive; S. cerevisiae was nonreactive; and C. albicans 4 and C.

Table 3. Agglutination reactivity of yeast strains with monoclonal antibodies C6 and H9 at 24 h growth.

	<u>STRAINS</u>	<u>H9</u>	<u>C6</u>
<u>C. ALBICANS</u>	94, 101, 102, 108, 122, 158, 160, 163, 164, 165, 169, 170, 1044	4+	4+
	105, 123, 159, 161, 168	3+	4+
	138, 166	1+	4+
	145, 157	2+	4+
	167	2+	2+
	174, 175	1+	1+
	1, 101, 9938	4+	3+
	394	-*	1+
<u>C. ALBICANS VAR. STELLATOIDEA</u>	171	1+	1+
<u>C. TROPICALIS</u>	4	4+	2+
	15	4+	4+
<u>C. PARATROPICALIS</u>	1	-	-
<u>C. PARAPSILOSIS</u>	1	-	-
<u>I. GLABRATA</u>	1, 15, 83-5	4+	-
<u>S. CEREVISIAE</u>	1	-	-

*C. ALBICANS 394 IS AGGLUTININ REACTIVE THROUGHOUT THE FIRST 18 H OF GROWTH AND BECOMES NONREACTIVE BY 24 H.

tropicalis 4 autoagglutinated in saline. Results of the growth curve studies indicated that the organisms formed 3 groups based on reactivity with H9 (Figure 2): 1) C. albicans 17 represents growth curve results obtained for C. albicans 4, C. albicans 19, and C. albicans 34 (Figure 2a), 2) C. albicans 394 formed the third group (Figure 2b), and 3) T. glabrata and C. albicans 9938 reacted similarly and the growth curves and reactivities of these 2 organisms are represented by the results for C. albicans 9938 (Figure 2c). With the exception of S. cerevisiae, all organisms, regardless of growth medium, exhibited transient autoagglutination (i.e., agglutination in saline and in uninoculated culture medium) in the first 2-6 h of growth. After 6 h autoagglutination occurred only in C. tropicalis 4. Autoagglutination of C. albicans 394 appeared to be transient and media-dependent, occasionally masking the observation of agglutinin reactivity in early log and late stationary phases (Figure 1b). At no time during growth was agglutinin reactivity observed in S. cerevisiae.

To compare the expression of the cell surface determinant C6 with determinant H9 during growth, growth curve experiments and semi-quantitative slide agglutination tests were done on 3 strains of C. albicans which included 1 representative of each of the 3 groups formed in previous agglutination-growth curve

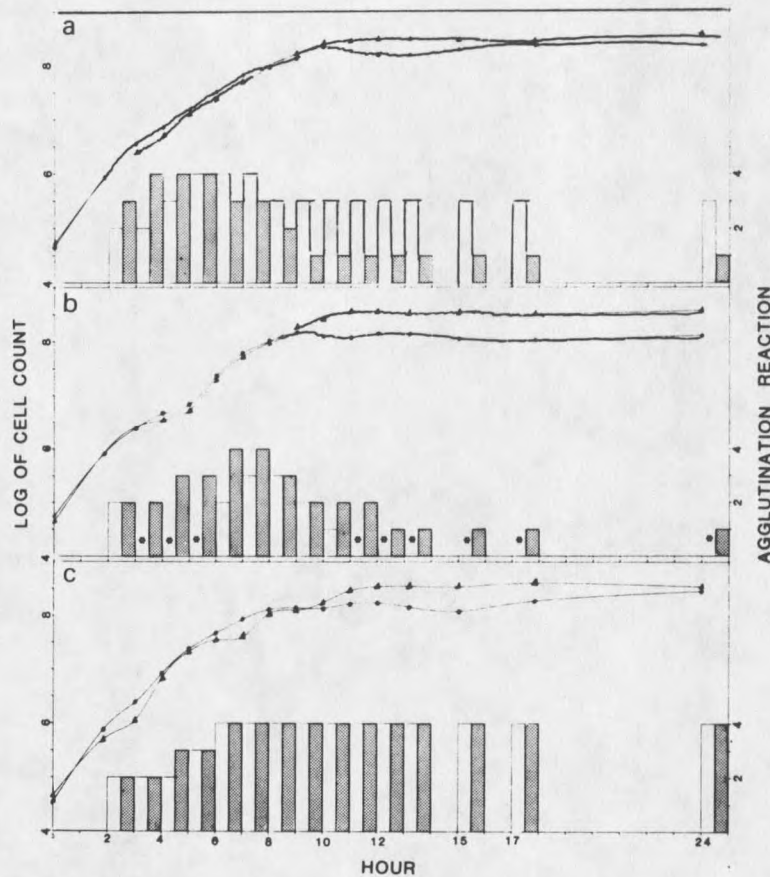


Figure 2. Growth-curve studies carried out on several yeast strains. Yeast cells were grown for 24 h at 37°C with rotation at 160 rpm in either GYEP broth or BHI broth. At hourly intervals, portions were removed from each broth, and the cells were counted (hemacytometer), washed, and tested for monoclonal agglutinin reactivity (agglutinin reactivity of GYEP broth-grown cells and reactivity of BHI broth-grown cells are indicated by stippled and unstippled bars, respectively). (a) Growth curves and agglutinin reactivity of *C. albicans* 17. (b) growth curves and agglutinin reactivity of *C. albicans* 394. (c) Growth curves and agglutinin reactivity of *C. albicans* 9938. Asterisks indicate autoagglutination of cells in saline and concentrated growth medium.

experiments utilizing H9 (Figure 2 and Table 1), and 1 strain of C. parapsilosis. Results are summarized in Table 4. In general, H9 agglutinin reactivity with C. albicans 9938 increased steadily from 2-4+ during early logarithmic phase, reached 4+ by 7 h, remained 4+ through 24h, and declined to 3+ by 48h. Monoclonal antibody C6 reacted similarly with C. albicans 9938 but agglutination peaked earlier and declined to 3+ by 10 h where it remained through 48 h. H9 agglutinated C. albicans 394 to 4+ by 6 h and declined to 1+ by 8 h. Reactivity was weak at 18 h and not detectable at 24 h. However, agglutination with C6 peaked by 5 h in this strain and remained at a high level throughout the 48 h period. In the third strain, C. albicans 105, H9 agglutination peaked early (5 h) and remained at this level until 10 h when it slowly declined from 3+ to 2+ by 48 h. Reactivity with C6, however, peaked at 8 h and remained 4+ throughout the entire 48 h. C. parapsilosis remained nonreactive throughout its entire growth cycle when tested for agglutination with either monoclonal antibody.

Effect of environment on agglutinin reactivity

Because agglutinin H9 reactivity of cells grown in GYEP differed from cells grown in BHI, additional studies were done to determine the effect of the nutritional environment on immunodeterminant expression. Yeast cells

Table 4. Growth curve agglutination and TEM comparisons of the amount of surface antigen on Candida albicans.

		C. ALBICANS 9938		C. ALBICANS 394		C. ALBICANS 105	
		AGGLUTINATION ^b	TEM ^c	AGGLUTINATION	TEM	AGGLUTINATION	TEM
4H:	C6	(*) ^a	VAR., -TO 4+	1+	VAR., -TO 3+	1+	3+
	H9	1+	VAR., 2+-4+	3+	3+-4+	3+	3+
6H	C6	1+		4+	VAR., -TO 4+	3+	
	H9	3+		4+	MOST 3+	4+	
8H	C6	3+		4+		4+	
	H9	4+		1+		4+	
10H	C6 ^d	4+	3+-4+ (DN)	4+	2+	4+	2+
	H9 ^d	4+	2+-3+	1+	2+	3+	SOME 4+, MOST 2+
18H	C6	3+		4+		4+	
	H9	4+		WK		3+	
24H	C6	3+	MOST 2+-3+	4+	MOST 3-4+	4+	2+
	H9	4+	- TO 2+	-	VAR., DN	3+	MOST-TO 2+
48H	C6	3+	MOST 2+-3+ (DN)	4+	SOME 4+, (MOST DN)	4+	
	H9	3+	MANY-, SOME 2+	-	MOST - AND DN	2+	

^a SYMBOLS. VAR=VARIABLE AMOUNT OF LABEL ON SURFACE FROM-TO+; DN=FIBRILLAR LAYER AND GOLD MARKER ARE DENUING FROM THE SURFACE OF THE CELLS; (*)=AUTOAGGLUTINATION.

^b AGGLUTINATION IN NORMAL ASCITES WAS NEGATIVE IN ALL CASES.

^c TEM EVALUATION. 1+=RARE (<10 PARTICLES OF GOLD/CELL); 2+=SCANTY (10-20 PARTICLES OF GOLD/CELL); 3+=MODERATE (20-100 PARTICLES OF GOLD/CELL); 4+=HEAVY(>100 PARTICLES OF GOLD/CELL); VAR=VARIABLE AMOUNT OF LABEL ON THE SURFACE FROM NONE TO MODERATE AMOUNTS.

^d AGGLUTINATION RESULTS ARE THE SAME AT 11H, 12H, 13H, AND 15H.

were generally more reactive with the agglutinin when the fungus was grown in broth media rather than on agar plates (Table 5). Enhanced agglutinin reactivity by BHI appeared to be dependent on actively growing cells because enhancement did not occur following incubation of cells in BHI at 4°C for 1 h.

Absorption and extraction procedures

Whole cell preparations were used to test the ability of individual yeast strains to absorb agglutinin H9 from hybridoma culture supernatant fluid. These results are compared to absorption tests which were done with soluble antigen extracts prepared from several of the same strains (Table 6). In general, strains which reacted strongly with the agglutinin completely absorbed out agglutinin reactivity whereas the capacity to absorb out the agglutinin could not be detected using either whole cells or soluble antigen extracts prepared from strains of yeast which were nonreactive. Whole cell and soluble antigen extracts prepared from weakly agglutinin-positive strains (*C. albicans* 394 and 19) did not reduce agglutinin titers significantly in absorption experiments. These results indicate that cells which do not agglutinate when mixed with the monoclonal antibody are, in fact, not expressing the antigen on their surface.

Table 5. Effect of *C. albicans* nutritional environment on agglutinin H9 reactivity ^a.

		<u>CANDIDA ALBICANS</u> STRAIN			
GROWTH MEDIA ^b		9938	17	34	394
BROTH	SAB	4+ ^c	-	1+	2+
	GYEP	4+	1+	1+	2+
	BHI	4+	3+	2+	3+(1+)
	PD	2+	1+(1+)	-	-
	SAAM	4+	1+	1+	-
	SAAMF	4+	3+	2+	-
AGAR	SAB	4+	-	-	-
	GYEP	4+	-	1+	-
	BHI	4+	1+	1+	-
	PD	2+	-	-	-
	SAAM	4+	2+	1+	-
	SAAMF	4+	1+	2+	-

^a FOUR STRAINS OF *C. ALBICANS* WERE GROWN IN VARIOUS BROTH OR SOLID MEDIA AT 37°C FOR 24 H, HARVESTED, COUNTED, WASHED IN SALINE, AND TESTED BY SLIDE AGGLUTINATION FOR REACTIVITY WITH THE H9 AGGLUTININ (AGGLUTININ TITER 128).

^b MEDIA INCLUDED SABOURAUD DEXTROSE (SAB), GLUCOSE YEAST EXTRACT PEPTONE (GYEP), BRAIN HEART INFUSION (BHI), POTATO DEXTROSE (PD), SYNTHETIC AMINO ACID MEDIUM (SAAM), SYNTHETIC AMINO ACID MEDIUM FOR FUNGI (SAAMF); EACH WERE EITHER PREPARED AS BROTH OR WERE SOLIDIFIED BY APPROPRIATE ADDITION OF AGAR (OR AGAROSE FOR SAAM AND SAAMF).

^c SLIDE AGGLUTINATION REACTIONS WERE SCORED FROM NO EVIDENCE OF AGGLUTINATION (-) TO MAXIMUM AGGLUTINATION (4+). AUTOAGGLUTINATION IN SALINE OR UNINOCULATED CULTURE MEDIUM OCCURRED IN ONLY TWO INSTANCES WHICH ARE INDICATED WITHIN PARENTHESES.

Table 6. H9 agglutinin absorptive ability of yeast strains.

ORGANISM USED FOR ABSORPTION ^a	AGGLUTINATION TITER ^b	
	AFTER WHOLE CELL ABSORPTION	AFTER SOLUBILIZED ANTIGEN EXTRACT ABSORPTION ^c
<u>C. ALBICANS</u> 9938	2	4
<u>C. ALBICANS</u> 394	32	128
<u>C. ALBICANS</u> 4	2	4
<u>C. ALBICANS</u> 19	16	N.D. ^f
<u>I. GLABRATA</u> 74	2	128
<u>S. CEREVISIAE</u> 78	32	128
<u>C. TROPICALIS</u> 4	2	N.D.
SALINE CONTROL	32 ^d	128 ^e

^a ORGANISMS USED FOR ABSORPTION STUDIES WERE GROWN IN BOTH BHI AND GYEP BROTH FOR 24 H AT 37°C. ABSORPTION RESULTS WERE IDENTICAL FOR ORGANISMS GROWN IN EITHER MEDIUM.

^b SEMI-QUANTITATIVE TUBE AGGLUTINATION TITERS WERE DETERMINED USING C. ALBICANS 9938 AS THE ANTIGEN. THE NUMBERS REPRESENT THE RECIPROCAL OF THE HIGHEST DILUTION OF AGGLUTININ WHICH RETAINED REACTIVITY.

^c WATER SOLUBLE EXTRACT OBTAINED FROM WHOLE CELLS FOLLOWING TREATMENT WITH 1 M NaCl AT 68°C.

^d MONOCLONAL ANTIBODY (POOLED CULTURE SUPERNATANT FLUID FROM CLONED HYBRIDOMA).

^e MONOCLONAL ANTIBODY CONCENTRATED BY (NH₄)₂SO₄ FRACTIONATION.

^f NOT DONE.

T. glabrata strain 74 agglutinated when mixed with the monoclonal antibody and the cells absorbed the antibody from solution. However, the antigen did not appear to be solubilized by hot NaCl since these extracts did not reduce the titer of a solution of the monoclonal antibody (Table 6).

FITC-labeled monoclonal antibody studies

To examine expression of the antigen on the surface of individual cells of C. albicans 9938, the cells were harvested from GYEP broth after various incubation periods and assessed for reactivity with the monoclonal antibody H9 by an indirect fluorescent antibody test.

At 5 h incubation, approximately 50% of the blastoconidia and 50% of the pseudohyphae fluoresced strongly (4+). Only about 30% of the cells examined at 24, 48, and 96 h incubation were reactive with the antibody and the fluorescence was weaker (2-3+). In addition to these observations, C. albicans 394, which produced weak agglutination in the presence of H9, also produced weak fluorescent reactions (1+ - 2+) by this test. These data support the concept that H9 antibody does not bind to cells which do not agglutinate.

Immunodot-spot serum assay for candidal antigenemia

The assay was able to detect antigens in (spiked) human sera in concentrations as low as 15 ng/ml. No antigen was detectable in the sera of moribund mice, and sera from 13 of the 14 patients colonized with C. albicans contained no antigens that could be detected by the monoclonal antibodies when using this method. One patient serum was weakly positive when B6 monoclonal antibody was tested but the test was inconclusive when repeated. Serum from the patient with disseminated candidiasis was also negative for antigens which reacted with B6, C6, or H9 monoclonal antibody.

Indirect transmission immunoelectron microscopy

Location of yeast phase surface determinants with ferritin-conjugated secondary antibody. High concentrations of monoclonal antibody (H9) in ascitic fluid and ferritin-conjugated secondary antibody were required to detect ferritin on the surface of the yeast cells. Binding was specific, however, in that cells treated with normal ascitic fluid and secondary antibody did not bind ferritin. Prior to fixation steps the yeast were embedded in 1% agarose which is an accepted and convenient method to alleviate many centrifugation steps during washing, dehydrating, and embedding procedures.

However, fixatives react with and stain carbohydrates which results in a dark background and poor resolution in EM micrographs and may have been the cause of the poor quality of some of our stained preparations. Ferritin was visible on cell surfaces (Figure 3) but often required magnifications of 50,000 to 60,000 for good resolution. This limitation precluded semiquantitative estimates of surface antigens because of the inability to visualize the entire cell periphery at these magnifications.

Location of yeast phase determinants with colloidal gold-conjugated antibody. Although autoagglutination which occurred with cells in early log phase growth prevented accurate detection of antigens on the surface of C. albicans strains 9938, 394 and 105 when agglutination techniques were used, immunolabeling of the surface antigens and electron microscopy established the presence of antigens at 4 h on these 3 organisms. The criterion for quantifying surface colloidal gold particles has been previously described (see Materials and Methods). C. albicans strains and C. parapsilosis treated with normal ascitic fluid and secondary antibody, however, were negative or bound <5 particles of gold per cell which ruled out nonspecific attachment of secondary antibody (Figure 4a). In general, at 4 h C. albicans 9938, when reacted with H9, showed variable surface expression; <10% of the cells were negative, 90% were scanty (2+) or



Figure 3. Ferritin-labeled C. albicans cell. C. albicans 9938 cell grown 12 h in GYEP broth, reacted with antibody H9 (titer = 6400), and a 1:2 dilution of ferritin-conjugated goat anti-mouse antibody. Magnification x116,000.

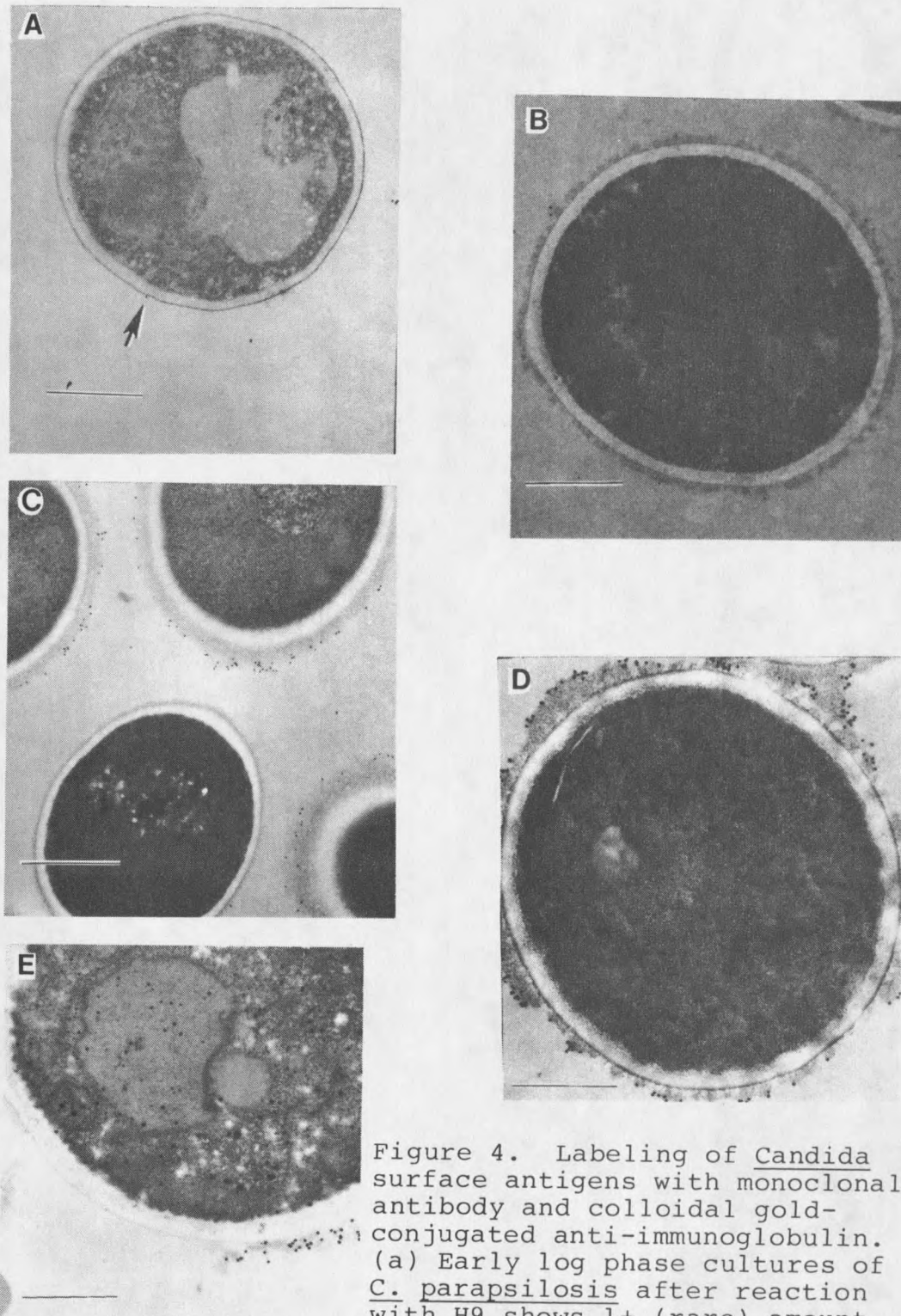


Figure 4. Labeling of *Candida* surface antigens with monoclonal antibody and colloidal gold-conjugated anti-immunoglobulin. (a) Early log phase cultures of *C. parapsilosis* after reaction with H9 shows 1+ (rare) amount of colloidal gold on the

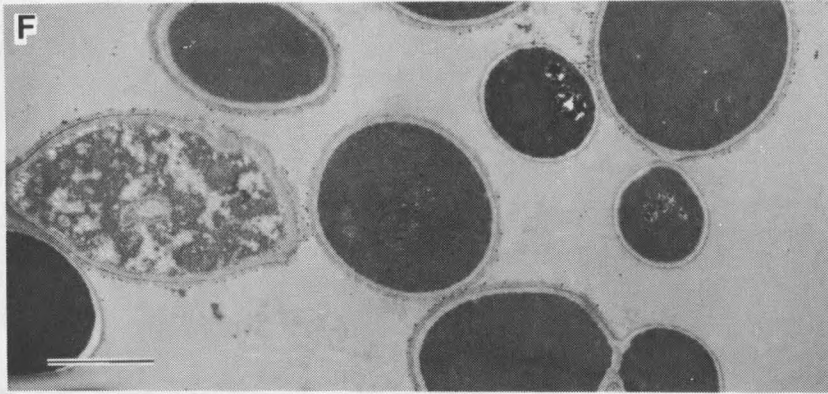


Figure 4 (cont'd)

surface; (b) a 10 h culture of C. albicans 394 when reacted with H9 binds 2+ (scant) gold to the cell surface; (c) C. albicans 105 at 4 h after reaction with C6 binds 3+ (moderate) amounts of colloidal gold to the surface; (d) C. albicans 9938 at 4 h after reaction with C6 binds 4+ (heavy) amounts of colloidal gold; (e) denuding of flocculent layer from the surface of a C. albicans 394 cell grown for 24 h and reacted with monoclonal antibody and gold-conjugated anti-immunoglobulin; (f) C. albicans 9938 in log phase after reaction with H9. Bars: 2 μ m (a-c); 1 μ m (d-f).

moderately (3+) labeled. At 10 h, although the agglutinin reactivity was still 4+, the amount of label on cells was scanty to moderate (Figure 4f). At 24 h, the results remained similar to those at 10 h except that approximately 25% of the cells appeared negative and by 24 h 75% of the cells appeared negative for colloidal gold and 25% were scant to moderately positive. When C. albicans 9938 was reacted with C6, by 4 h >90% of the organisms were heavily labeled (Figure 4d) which dropped to moderately positive by 10 h. At 24 and 48 h surface label was scant to moderate. Ninety percent of C. albicans 394 were moderately to heavily positive at 4-6 h when reacted with H9 and secondary antibody. Cells were scanty labeled at 10 to 24 h (Figure 4b) and antigen was denuding from the surface. At 48 h >95% of the cells appeared negative. When reacted with C6, half of the yeast were scanty labeled at 4h although variability in the amount of label ranged from negative to moderate in the remaining cells. By 6h >90% of the cells were moderately labeled, <5% were negative and an occasional cell was heavily labeled. By 12 h nearly 100% of the cells were scanty to moderately positive. At 24 h all cells were moderately to heavily positive but showed signs of denuding their antigen surface by 48h (Figure 4e). Cells of C. albicans 105, when reacted with H9, were moderately positive at 4 h (Figure 4c) but the amount of label (when

present) was scanty at 10 and 24 h. As many as 50% of the cells also appeared negative at this time but an occasional cell (<5%) was also heavily labeled. When reacted with C6, this organism was moderately positive at 4 h and became weakly positive at 10 h and 24 h with a rare (ca. 1%) heavily labeled organism. Neither the antigens nor the flocculent layer was expressed on surfaces of young birth scars or during early stages of bud formation (Figure 5a and c), but with maturity, bud scars expressed the flocculent layer and bound colloidal gold (Figure 5b).

Preabsorption of diluted mouse ascitic fluid.

Absorbed normal ascitic fluid-treated cells were compared with unabsorbed normal ascitic fluid treated-cells by immunoelectron microscopy to determine if the nonspecific adherence of colloidal gold-conjugated antibody observed in normal ascitic fluid control cells was due to extraneous antibody in the normal fluid (other than monoclonal antibody). Normal ascitic fluid was absorbed with both GYEPB and GM-2 grown cells as described. Despite treatment with absorbed normal ascitic fluid, cells nonspecifically bound colloidal gold conjugated antibody.

Treatment to block nonspecific adherence of gold-conjugated antibody. Germinating cells that were treated with undiluted fetal bovine serum (FBS) or 20% bovine

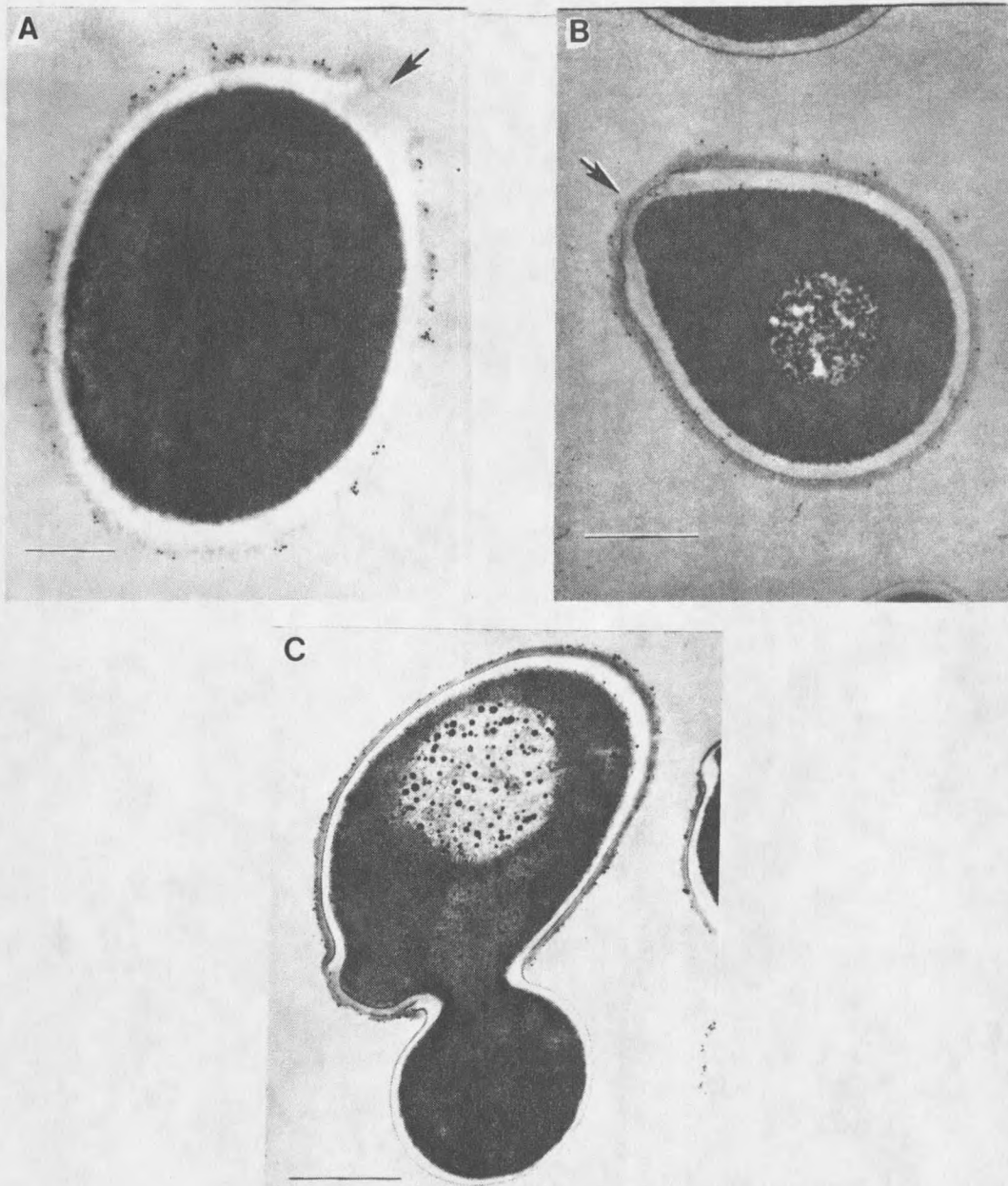


Figure 5. Variable expression of gold-labeled antigens of surface features of *C. albicans*. (a) A birth scar on *C. albicans* 9938. Arrow indicates absence of flocculent layer. Gold label appeared on more mature bud scars (b); (c) the flocculent layer was not present on surfaces of buds, but mother cells expressed the flocculent layer and bound gold (10 h culture of *C. albicans* 105 after treatment with H9). Heavy metal precipitate from post-section stains is visible within cell (c). Bars = 1 μ m.

serum albumin (BSA) solution exhibited neither reduced autoagglutination nor less nonspecific binding of colloidal gold to germ tubes when compared to untreated (germinating) cells.

Trypsin treatment of germinating and nongerminating cells. Trypsinization of germinating cells eliminated autoagglutination of cells at 1, 2, and 4 h germination and reduced the degree of autoagglutination at 20 h germination. In general, nongerminating cells did not autoagglutinate or bind gold-labeled antibody nonspecifically and trypsinization did not diminish the ability to bind gold after treatment with monoclonal antibody, in that when populations of trypsin-treated cells and corresponding nontrypsinized controls from all time periods were prepared for immunoelectron microscopy and examined, both cell populations when reacted with H9 or C6 and secondary antibody bound scant (2+) to moderate (3+) gold (Figure 6a and 6b).

Germinating cells which had consistently bound gold label nonspecifically without trypsin treatment (Figure 6c), bound very little (1+) if any gold nonspecifically after trypsin treatment (Figure 6d). Although electron micrographs (shown in Figure 6) represent cells grown for 2.5 h, cells at all time periods (1, 2, 4 and 20 h) as well as cells treated with either H9 or C6 monoclonal

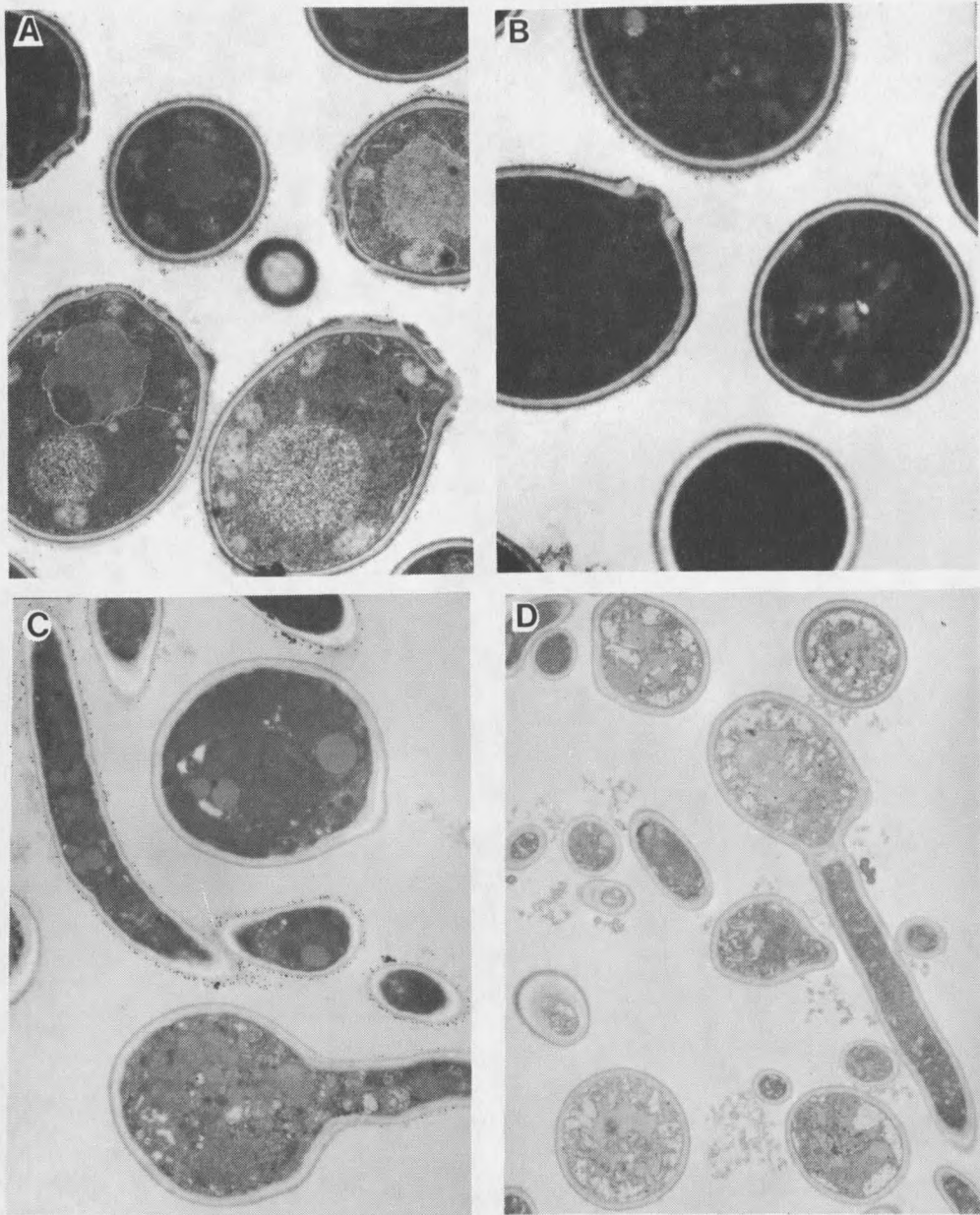


Figure 6. Effects of trypsin treatment on specific and nonspecific binding of gold-conjugated antibody. Trypsin treatment had no effect on binding of gold to nongerminating cells. Yeast phase cells grown 2.5 h which were either trypsin-treated (a) or untreated (b), both bound scant to moderate gold. (After enzyme-treatment, both cell populations were reacted with H9 and gold-conjugated anti-Ig). Germinating cells nonspecifically bound gold-conjugated antibody to germ tube surfaces. At

Figure 6 (cont'd)

2.5 h, germ tubes of untreated cells (c), reacted with normal ascitic fluid and colloidal gold-conjugated anti-Ig bound label heavily whereas germ tubes from cells pretreated with trypsin prior to reactions with normal ascitic fluid and gold-conjugated antibody were rarely labeled or negative (d). Magnification (a) and (c) x12,000; (b) x14,500; (d) x13,000.

antibody in nongerminating populations at 0, 15, and 30 min reacted similarly.

Variability of surface antigens during morphogenesis.

Table 7 summarizes the results of the antigenic variability studies of C. albicans 105 in both germinating and nongerminating cells. Seventy-five percent of germinating and nongerminating cells reacted with H9 at 0 and 0.25 h incubation, and bound colloidal gold at a 2-3+ level. Germ tubes, first apparent at 1 h, bound 2+ gold but mother cells were unlabeled (Figure 7c). Nongerminating cells observed at 1 h varied in the amount of H9 specific-antigen expressed; 75% of the cells were either 3 or 4+ labeled while others were only 1 or 2+ labeled. By 2 h germinating mother cells were unlabeled and germ tubes were labeled 2-3+, most heavily at the apices (Figure 7d), while 95% of nongerminating cells were 2+ or 3+ labeled. At 4 h approximately 1% of the mother cells had re-expressed small amounts (1-2+) of surface antigens (Figure 7e). Nongerminating cells remained essentially unchanged. By 20 h, all mother cells were re-expressing a large quantity (4+) of H9-specific antigen and germ tubes retained 2+ label (Figure 7f).

Washed cells used to inoculate GM-2 (0 h) when reacted with C6 were all labeled 1-2+. Nongerminating cells observed at 0.25 and 0.5 h expressed antigens variably (Figure 8a). Seventy-five percent of the cells were

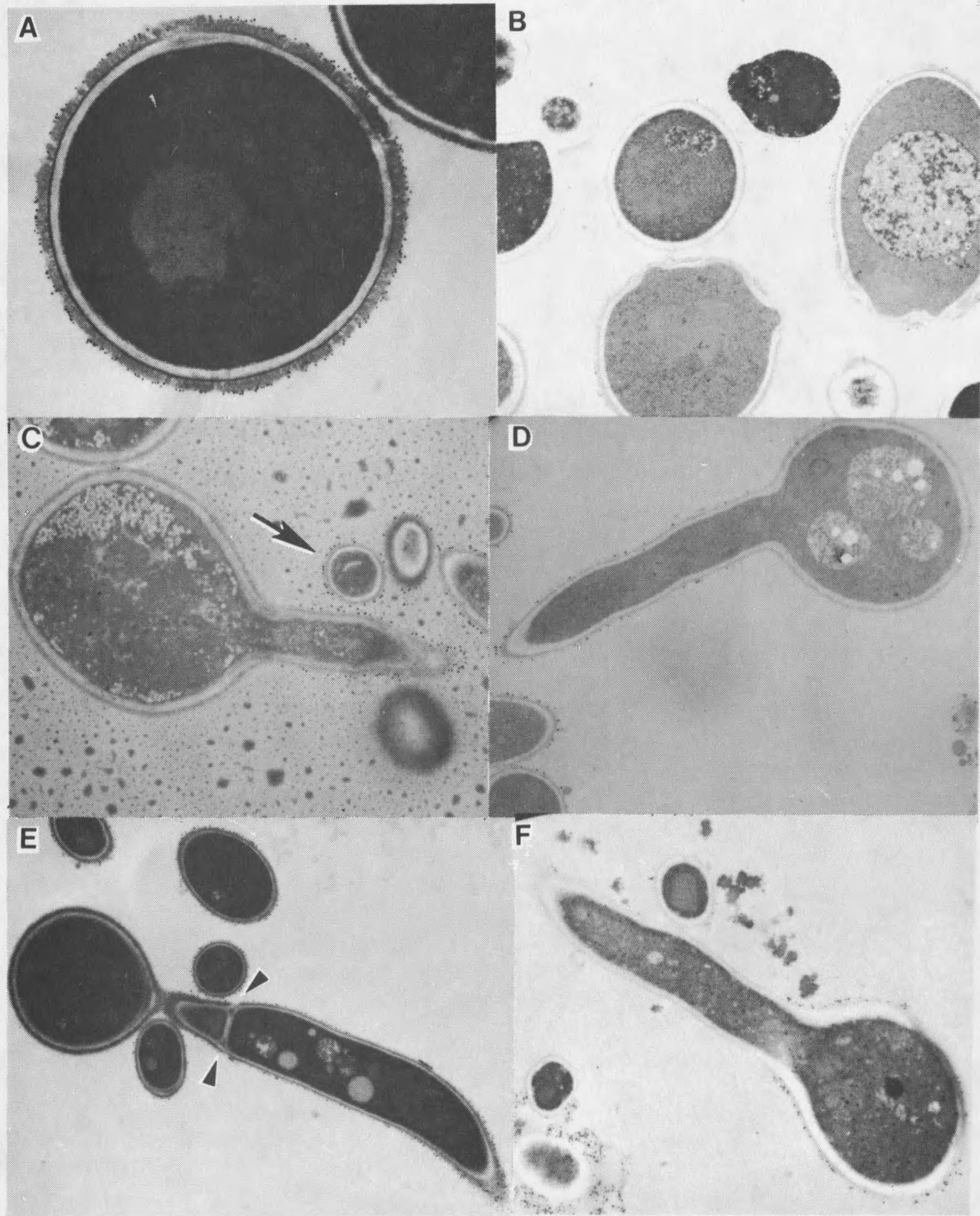


Figure 7. Variable expression of antigenic determinants specific for monoclonal antibody H9 during morphogenesis of *C. albicans* 105. (a) yeast cells suppressed from

Figure 7. (cont'd)

germinating in GM-2 exhibit variability in amount of label bound. At all time periods tested (15 min through 4 h) a few cells were negative or heavily labeled, but most cells bound scant (2+) to moderate (3+) gold. (b) AgH9 expression varied on cells examined at both 0.25 and 0.5 h germination. Most cells bound gold scanty (2+) and <10% were negative; (c) by 1 h germination, mother cells rarely bound gold but germ tubes were positive and most heavily labeled at the apices; at 2 h and 4 h germination (d and e, respectively), mother cells remained negative and germ tubes were positive for AgH9, but by 20 h mother cells resumed AgH9 expression and were moderately to heavily labeled while germ tubes appeared to retain only scanty amounts of label (f). Arrows in (e) indicate cross sections of hyphae. Magnification: (a) x25,000, (b) and (d) x13,000, (c) and (f) x12,000, and (e) x9500.

Table 7. Detection of surface antigens of Candida albicans by indirect immunoelectron microscopy.

TIME PERIOD		GERMINATING CELLS		NONGERMINATING CELLS ^A	
		ANTIBODY H9	ANTIBODY C6	ANTIBODY H9	ANTIBODY C6
0.25		2+ ^B (75%) ^C	2+ (99%)	2-3+ (75%)	3-4+ (25%) ^E
0.50		2+ (99%)	2-3+ (100%)	ND	ND
1.0	MOTHER CELL	- (100%)	2-3+ (100%)	3-4+ (75%)	1-2+ (100%)
	GERM TUBE	2+ ^D (100%)	1-2+ ^D (100%)		
2.0	MOTHER CELL	- (100%)	3+ (100%)	2-3+ (95%)	1-2+ (100%)
	GERM TUBE	2+ (100%)	1-2+ (100%)		
4.0	MOTHER CELL	- (99%)	3+ (100%)	2-4+ (75%)	1-3+ (10 %)
	GERM TUBE	1-2+ (100%)	1-2+ (100%)		
20	MOTHER CELL	4+ (100%)	ND ^F	ND	ND
	GERM TUBE	2+ (100%)			

^A MARS TREATED OR A HIGH YEAST CELL CONCENTRATION

^B TEM EVALUATION: 1+ = RARE (<10 PARTICLE OF GOLD BOUND/CELL); 2+ = SCANTY (10-20 PARTICLES OF GOLD BOUND/CELL); 3+ = MODERATE (20-100 PARTICLES GOLD BOUND/CELL); 4+ = HEAVY (>100 PARTICLES OF GOLD BOUND/CELL); - = USUALLY 0 GOLD PARTICLES/CELL, RARELY UP TO 3

^C PERCENT OF CELLS DISPLAYING INDICATED LABEL DENSITY

^D MOST HEAVILY LABELED AT APICES

^E REMAINING 75% WERE - OR 1+

^F ND - NOT DONE

either 1+ labeled or negative while approximately 25% were 3-4+ labeled (Figure 8b). In marked contrast to results obtained with the H9 antibody, all germinating mother cells were reactive with C6 antibody at 1, 2, and 4 h incubation (Figure 8c, d, e). In addition, nongerminating cells incubated for 1 h or longer consistently reacted less intensely with C6 antibody than with antibody H9 (Table 7).

Scanning electron microscopy

Enzyme-linked antigen distribution assay (ELADA) to quantify surface antigens. Although the Miles-Yeda phosphatase-conjugated enzyme was reactive in control tests when substrate was added, no insoluble substrate-enzyme complexes could be seen on the surface of Candida cells by SEM regardless of attempts to vary the thickness of the gold sputter-coating from 3 min at 20 milliamps to 1 min at 20 ma (range from <1 to 65 nm thickness). Light microscopy of the phosphatase-reacted yeast cells was equivocal, i.e. a few cells when viewed with oil immersion appeared to have blue spots unevenly distributed on the surface.

Background electron imaging of C. albicans cells treated with gold-conjugated antibody. When SEM and BEI images were superimposed on the JEOL 300X scanning microscope at 10 keV, gold particles were viewed on a 3

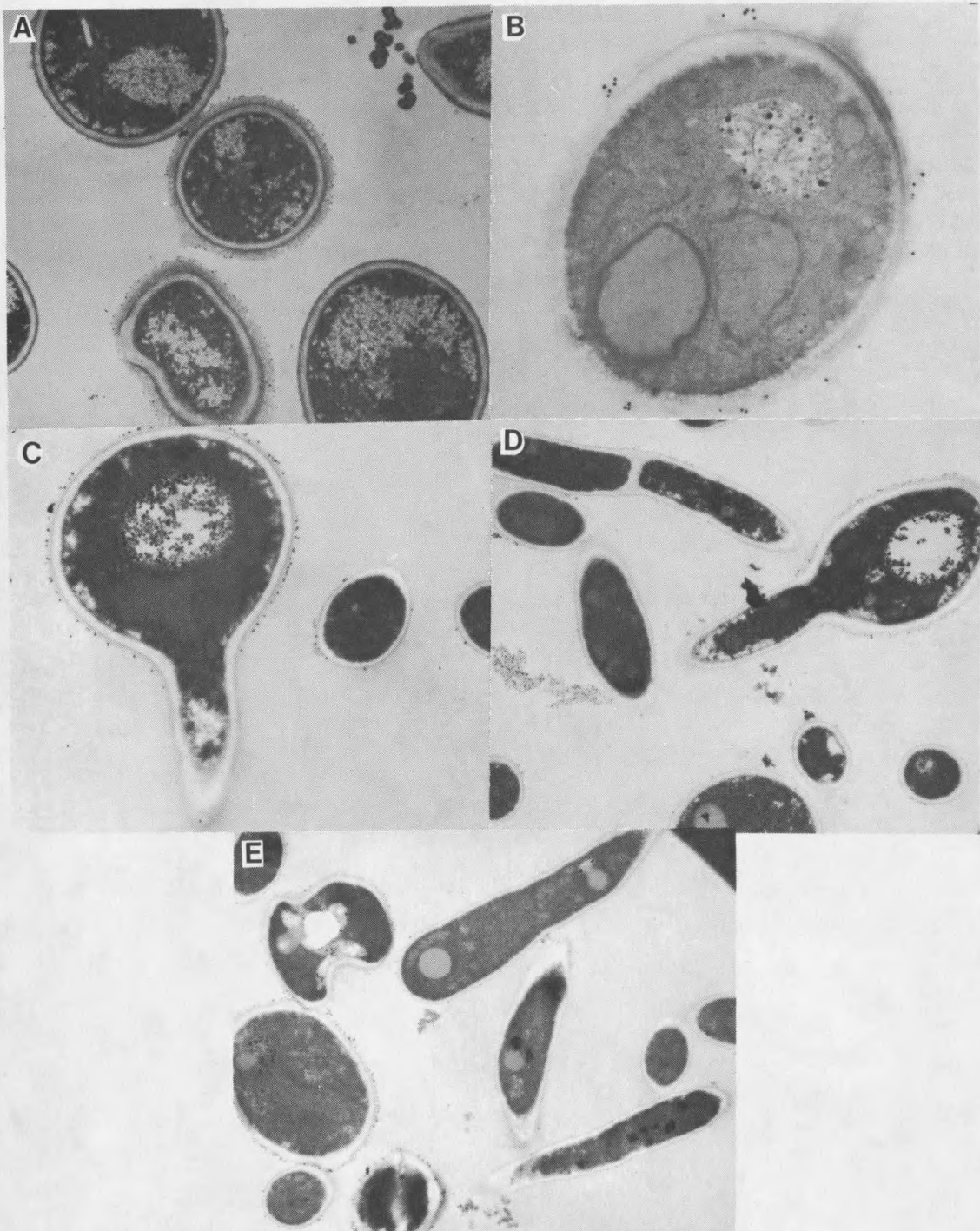


Figure 8. Variability in expression of the antigenic determinant for antibody C6 during morphogenesis of *C. albicans* 105. (a) yeast cells suppressed from

Figure 8. (cont'd)

germ tube formation varied in AgC6 expression (negative to moderate (3+) amounts of label bound) during all time periods tested (Table 1), (b) antigen expression was scanty (2+) to moderate (3+) on cells examined at 0.25 and 0.5 h germination, (c) at 1 h germination, mother cells and germ tubes expressed scanty to moderate amounts of AgC6; (d and e) at 2 and 4 h mother cells expressed moderate amounts of AgC6 while germ tube expression was rare to scant. Magnification: (a) and (c) x12,000; (b) x30,000; (d) x9,500; and (e) x13,000.

dimensional surface of Candida cells. The gold particles could be resolved at low magnification/low voltage on the SEM alone (Figure 9a and b), but resolution was lost at higher magnification due to increased voltage. Unfortunately, the STEM microscope available at MSU is unable to resolve images when less than 40 keV are used. Therefore, either larger colloidal gold particles would be needed in the study which could create steric hindrance problems or access to a scanning electron microscope equipped with a background detector would be necessary to complete an accurate quantification of surface antigen.

Topographical surface changes of cells during germination. Yeast cells grown to stationary phase in GYEP broth were inoculated into germinating medium (GM-2) and no attempt was made to synchronize germination. Instead, assessments were based on the relative length of the germ tube which can be used as a relative basis of comparison in these studies because growth kinetics of germlings are linear (46). Cells examined 15 min to 1 h into germination were entire and showed no signs of collapse despite the fact that some of the cells had germinated (Figure 10a). This was also true of cells which had been exposed to germinating conditions for 1 to 2 h. The length of the germ tubes had continued to increase but the cell walls were not collapsed (Figure 10b). However, cells which had been subjected to

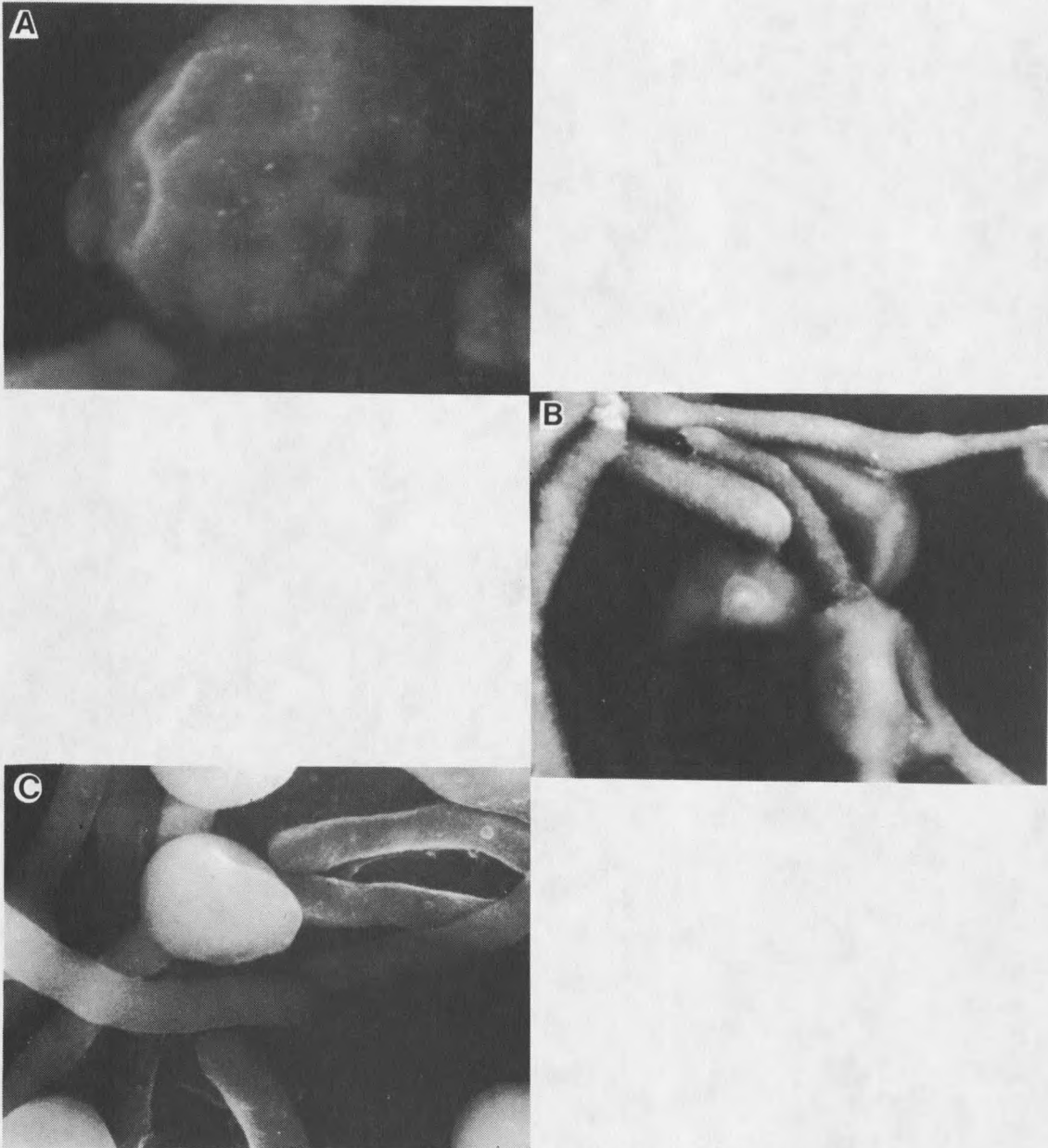


Figure 9. BEI photomicrographs of *C. albicans* 105 (a) nongerminating cell reacted with monoclonal antibody C6 and colloidal gold-conjugated anti-Ig (x20,000); (b) germinating cells grown 10 h in GM-2 and reacted with C6 antibody and gold-conjugated anti-Ig (x10,000); (c) germinating cells after 10 h in GM-2 reacted with normal ascitic fluid and gold-conjugated anti-Ig (x10,000).

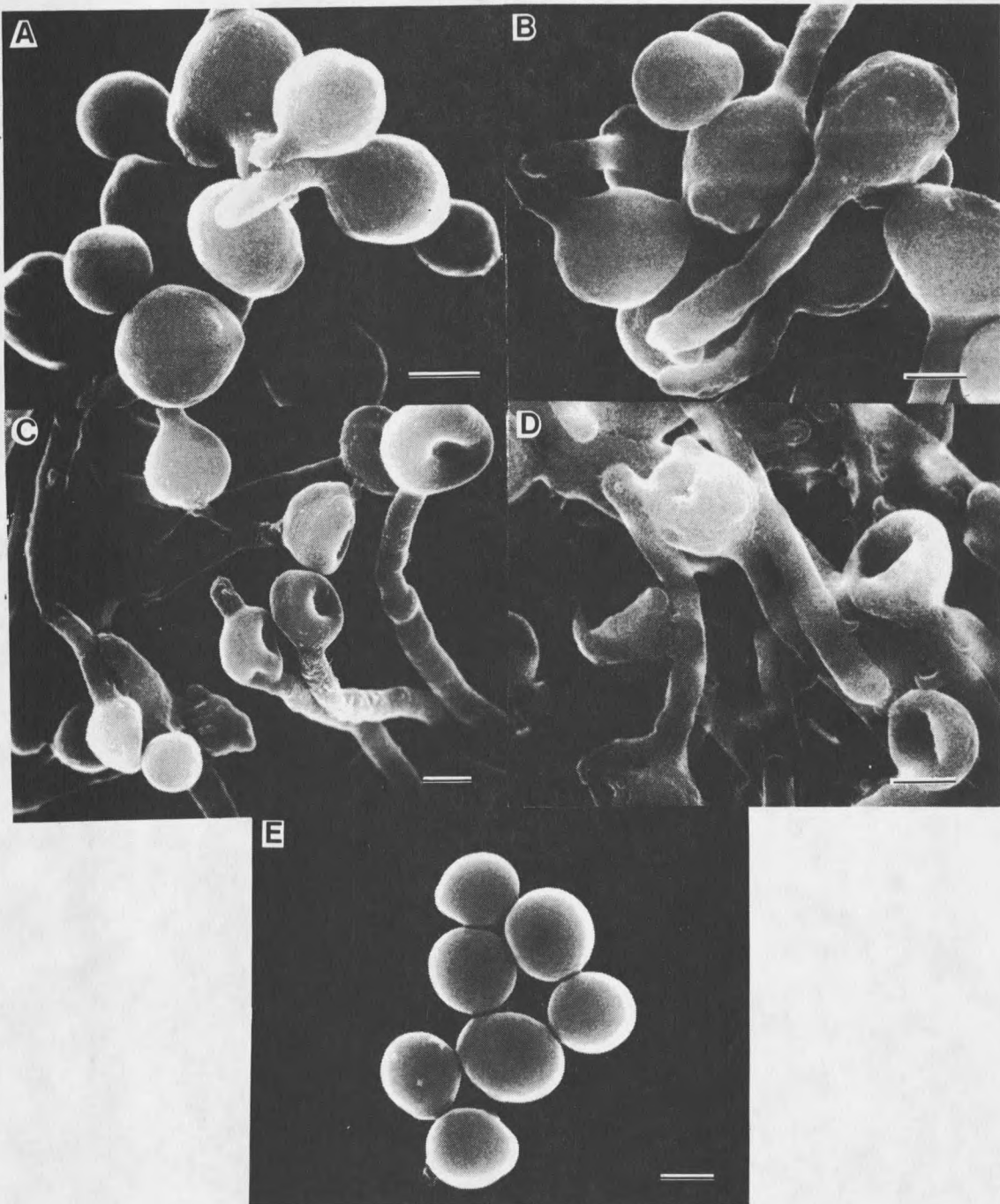


Figure 10. Germinating mother cells of *C. albicans* 105 examined at 0.25 to 1 h (a) and 1-2 h (b) are not

Figure 10 (cont'd)

collapsed. Those examined at 4 h (c) and 8 h (d) are collapsed. At 8 h when germination was suppressed, cell walls did not collapse (e) after the same treatment as (a-d). Scale bars represent 2 μ m.

germinating conditions for periods of 4 h or more (Figure 10c and d, respectively) showed marked tendencies to collapse while comparable cells which had been grown under nongermination conditions at no time showed signs of collapse (Figure 10e). Occasionally, cells which had been embedded in Spurr's resin also were collapsed when examined by TEM at time intervals which corresponded to those examined using SEM. Collapse of the cell walls occurred near the area of the cytoplasm which contained the vacuole (Figure 11a). Yeast cells which had been grown under nongerminating conditions and subsequently fixed and concentrated onto nucleopore filters showed evidence of flexible cell walls (Figure 11b).

Characterization of antigens in crude antigen extract

After heating crude antigen extract at 95.5°C for 40 min, it remained reactive with monoclonal antibodies C6 and H9. After precipitation of the antigen by either cold ethanol or TCA, reactivity to both monoclonal antibodies was determined by resolubilizing the precipitates in saline, dialyzing against saline, and testing for antigens by Ouchterlony double diffusion. Material not precipitated by ethanol and TCA was exhaustively dialyzed and also tested for the presence of antigen. Antigens were found in cold ethanol-precipitated fractions and in TCA supernatant fluid fractions, but

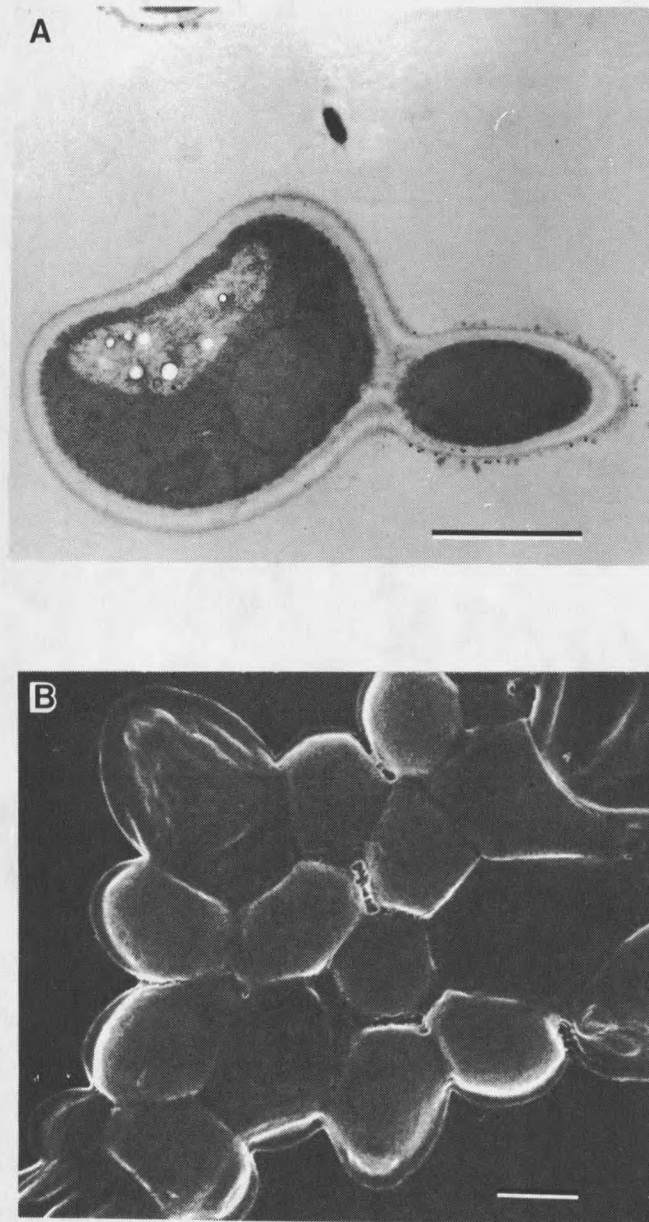


Figure 11. TEM photomicrograph demonstrates that at 2.5 h germination collapse of cell wall follows the contours of the vacuole beneath it (a). Yeast cells grown in nongerminating conditions and concentrated on nucleopore filters have flexible cell walls (b). Scale bars = μm .

antigens did not precipitate at any concentration of $(\text{NH}_4)_2\text{SO}_4$ tested (33-75%).

Protein and carbohydrate determinations on crude antigen extract were 29.4% and 20%, respectively. When 10% vertical SDS-PAGE was run on antigen obtained from hot 1 M NaCl treatment (as described above), Coomassie blue staining of the gels revealed 2 protein bands, one at the top and another at 6.8 cm or approximately 45 kd as determined by protein standards. However, stained bands were also visible between the bromphenol blue indicator and protein fronts which could not be identified. SDS-PAGE (10%) gels for carbohydrate were run simultaneously from the same solution of antigen extract and stained with Schiff reagent. These gels revealed only a dense band at the top of the gel indicating that the carbohydrate did not migrate into the gel. When IEF flat bed gels were run under non-reducing conditions, gels stained for protein (Coomassie blue) showed only a dense blue stain around the well indicating the proteins did not migrate from the well. Gels stained for carbohydrate (Schiff reagent) contained 2 bands which migrated toward the anode (2.0 and 1.5 cm, respectively) and one which migrated toward the cathode (2.5 cm). Fetuin, a glycoprotein (Sigma, MW. = 50,700) was run as a control and migrated approximately 2.5 cm toward the cathode. Therefore, the crude extract

contains at least 3 carbohydrate moieties which can be separated by charge.

Isolation of antigens from antigen extract

As stated earlier, attempts to purify antigens by affinity chromatography were unsuccessful probably because of the strong affinity of the monoclonal antibodies for their antigens. Therefore, we attempted to extract antigen from precipitated antigen-antibody complexes by Pronase B (protease) digestion.

When tritiated monoclonal antibodies (C6 and H9) and their respective antigens were reacted, and subsequently protease-treated and dialyzed, the labeled antibody was effectively removed from the antigen after enzyme treatment as indicated by the decrease in average cpms (Table 8). Antigen and labeled antibody were reacted empirically in this experiment because the expense of producing large quantities of tritiated antibody, needed to determine optimal antigen-antibody ratios, was prohibitive. After incubation the mixture of antigen and antibody was centrifuged and no pellet was observed. Supernatant fluid was treated as previously described (Figure 1). Subsequent protease treatment, centrifugation, and dialysis reduced counts to levels which were not significantly higher than background levels. When Ouchterlony tests were done on protease-

Table 8. Evidence that protease treatment removes antibody from antigen-antibody complexes. ^a

REACTANTS	AMOUNT OF ANTIBODY (CPM)			
	BEFORE PROTEASE OR DIALYSIS (FRACTION S) ^B	BEFORE PROTEASE, AFTER DIALYSIS (S _d)	AFTER PROTEASE, BEFORE 2 ND DIALYSIS (s/s)	AFTER PROTEASE, AFTER 2 ND DIALYSIS (d s/s)
³ H-H9 ANTIBODY + ANTIGEN ^C	204,168	25,500	10,235	42
³ H-C6 ANTIBODY + ANTIGEN	182,580	13,512	9,928	47
AMINO ACID FREE MEDIUM + ANTIGEN	39	46	46	39
UNLABELED H9 ANTIBODY ^D + ANTIGEN	44	45	78	110
³ H-AMINO ACID MIXTURE ^E + ANTIGEN	424	141	63	39
AMINO ACID FREE MEDIUM ^F	629	46	39	38
OPTISOL ONLY ^G	56	41	42	39

^A ALL DETECTABLE ANTIGEN WAS FOUND IN FRACTION ds/S (SEE FIG. 1) AS DETERMINED BY OUCHTERLONY TESTS.

^B SEE FIG. 1 FLOW DIAGRAM FOR DESIGNATION OF FRACTIONS COUNTED. S = SUPERNATANT FLUID, D = DIALYSED FLUID.

^C 2.0 MG CRUDE ANTIGEN EXTRACT + 1.0 ML ³H-ANTIBODY (LABELED CELL SUPERNATANT FLUID).

^D 1.0 ML UNLABELED H9 CELL CULTURE SUPERNATANT FLUID

^E 10 UL REAGENT + 2.0 MG CRUDE ANTIGEN EXTRACT

^F 10 UL AMINO ACID FREE MEDIUM IN 3.0 ML OPTISOL

^G 3.0 ML OPTISOL ONLY

treated, dialyzed supernatant fluid and pellet (ds/s and ds/p), antigen was found only in the ds/s fraction which indicated that antigen was resistant to Pronase B treatment and that this method was effective in isolating the antigen from antibody. A large quantity of precipitate was needed to obtain a sufficient amount of antigen for analysis. Therefore, antibody was combined with antigen at optimal proportions as described in Materials and Methods. In initial determinations of optimal proportions 10 mg of crude antigen was combined with 1.0 ml H9 antibody in ascitic fluid (titer = 25,000) and 8.8 mg of crude antigen was combined with 1.0 ml of C6 antibody (titer = 6400). Subsequently, approximately 150 ml of H9 ascitic fluid and 150 ml of C6 ascitic fluid were combined with 1.5 g and 1.0 g of crude antigen extract and protease-treated to produce 32 mg of H9 and 30 mg of C6 "isolated" antigen.

Characterization of isolated antigens C6 and H9

Lipid analysis by thin layer chromatography of hexane extracted fatty acids did not detect lipids in either crude antigen, isolated AgC6 or isolated AgH9.

When 5 mg/ml suspensions of crude antigen extract, AgC6, and AgH9 were examined by UV spectrophotometry, the scan of crude extract resulted in a large, broad peak from 185-295 nm (Figure 12) while UV scans of isolated antigen

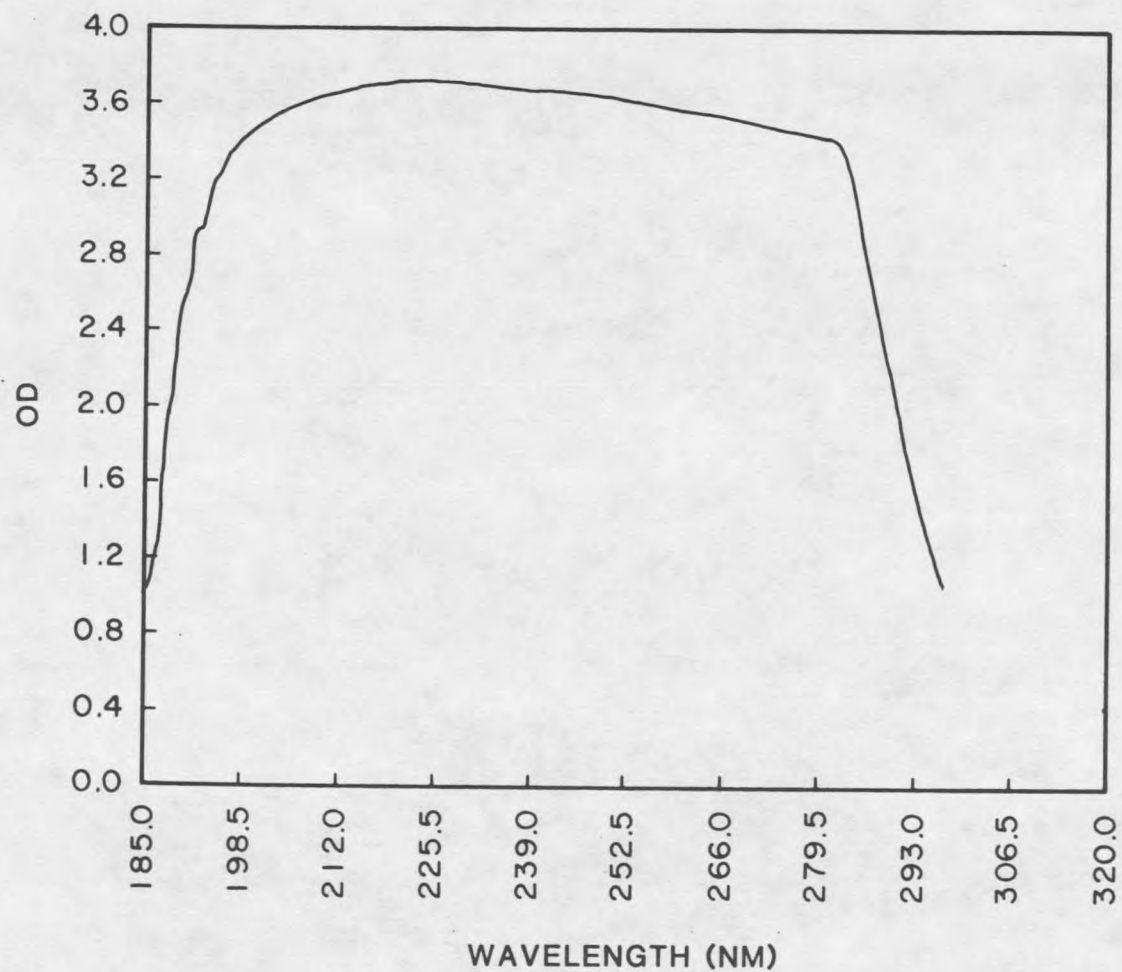


Figure 12. UV spectroscopy of a 5 mg/ml solution of crude antigen extract. The scan was done on a Gilford 250 spectrophotometer and absorbance was monitored from 185-295 nm.

suspensions showed peaks at 218 and 221 nm, for AgC6 and AgH9, respectively (Figures 13 and 14). HPLC fractions were collected and monitored either by UV detection during fraction collection (254 nm, Ultrasphere ODS column) or by UV spectrophotometry at 220 nm in the Gilford 250 spectrophotometer (fractions collected from the Spherogel 3000 TS-K column). Of the 2 fractions collected from the Ultrasphere column, only the first fraction contained antigens which reacted with antibodies C6 and H9. For the first time, double bands forming overlapping bands of identity (as shown in Figure 15), were noted in Ouchterlony double diffusion tests, indicating cross-reactivity which had not been discernible in previous Ouchterlony tests. These double bands remained visible in all subsequent Ouchterlony tests and were confirmed by immunoelectrophoresis of the antigens (Figures 16 and 17). Fractions 1 and 2 of AgC6 and AgH9 (isolated from the Ultrasphere ODS column) were eluted in 1.0 ml fractions from the steric exclusion column (Spherogel 3000 TS-K), scanned for UV absorbance at 220 nm, pooled (based on peaks), and tested by Ouchterlony diffusion tests. The majority of the antigen was found in fractions 5-8 (AgH9) and 6-8 (AgC6) as shown in Figure 18 and 19. Very small amounts of AgC6 and AgH9 were contained in the pooled fractions 5-15 of fraction 2 in both cases as determined by very weak diffuse bands which reacted with antibodies

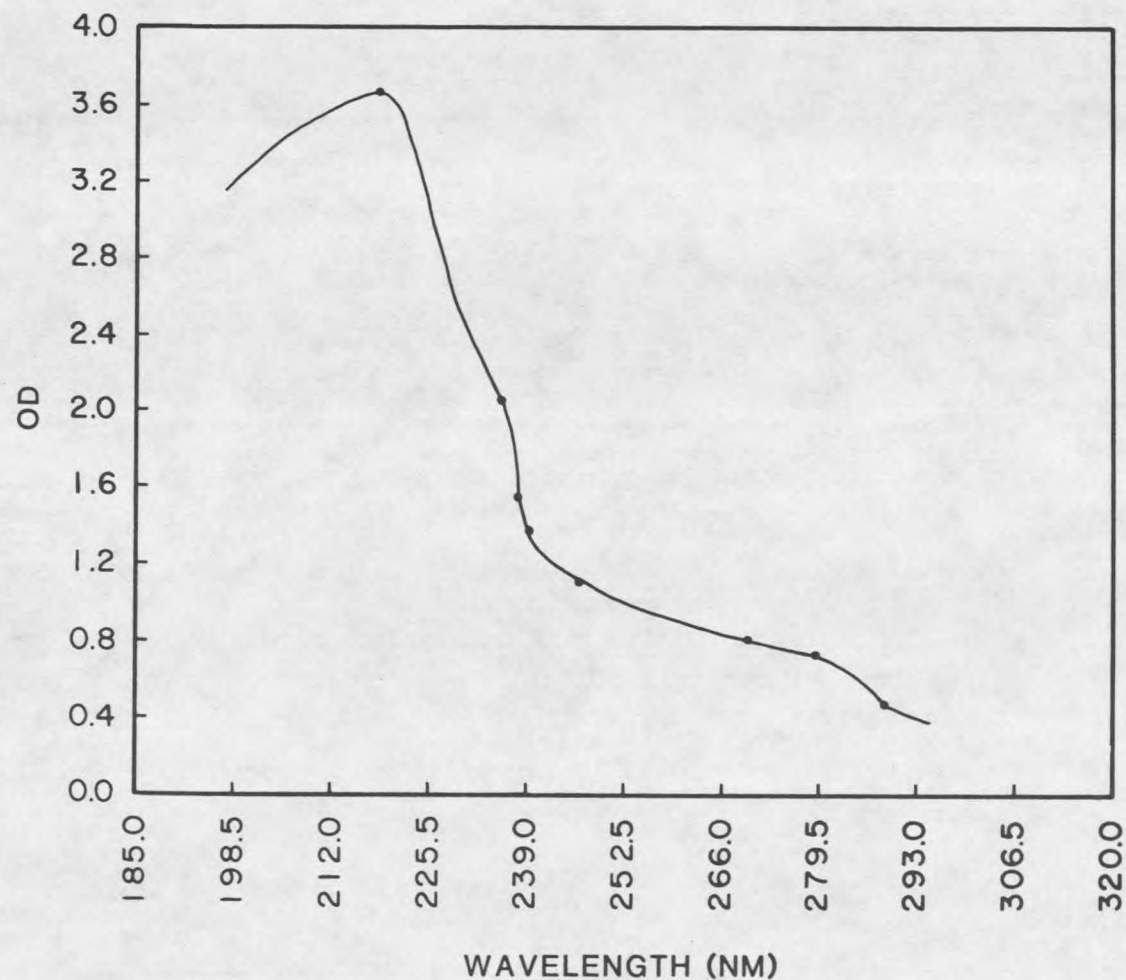


Figure 13. UV spectroscopy of a 5 mg/ml solution of clarified AgC6. The scan was done on a Gilford 250 spectrophotometer and absorbance was monitored from 198-295 nm.

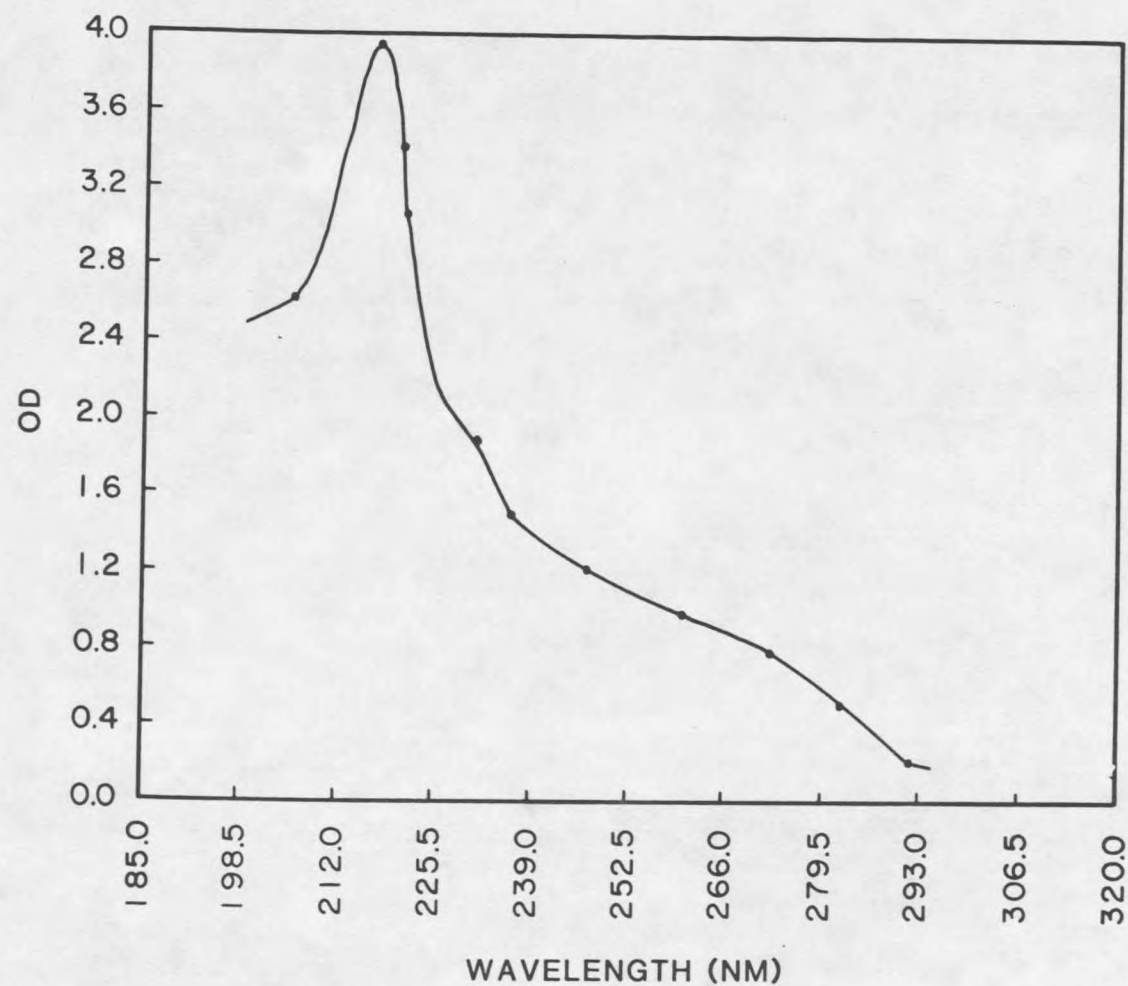


Figure 14. UV spectroscopy of a 5 mg/ml solution of clarified AgH9. The scan was done on a Gilford 250 spectrophotometer and absorbance was read from 198-295 nm.

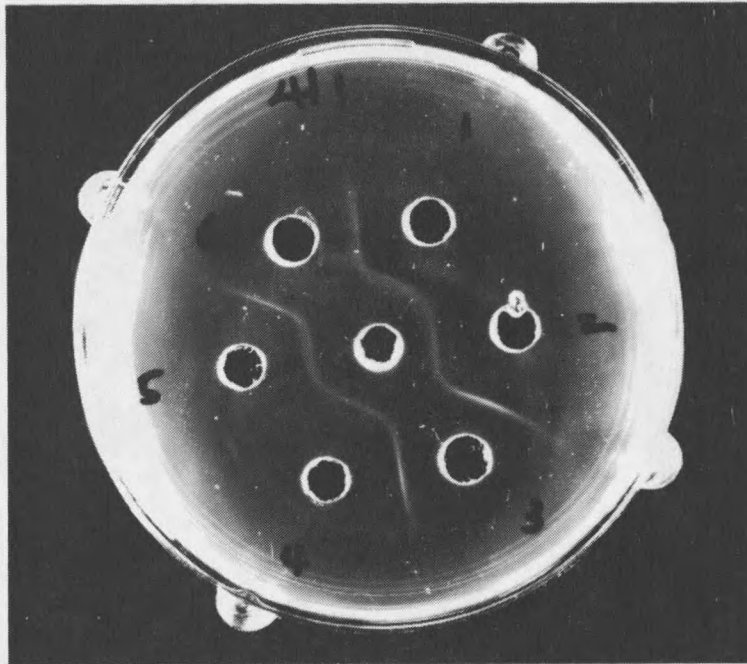


Figure 15. Ouchterlony double diffusion tests of HPLC-isolated AgC6 and AgH9 reacted with antibodies C6 and H9. Double overlapping bands of identity can be seen. Well #1 contained AgC6 (fraction 1); well #2 contained AgH9 (fraction 1); well #3 contained H9 antibody (titer = 12,800); well #4 contained AgH9 (fraction 2); well #5 contained AgC6 (fraction 2); and well #6 contained C6 antibody (titer = 3200). The center well contained equal quantities of C6 and H9 antibodies. All wells were loaded with 18 μ l of sample.

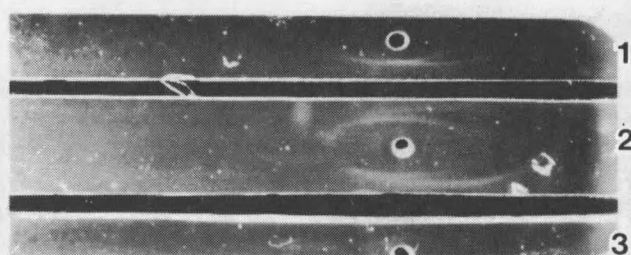


Figure 16. Immunoelectrophoresis of HPLC-isolated antigens. AgC6 and AgH9 (ca. 3 μ l of a 5 mg/ml solution) were loaded into wells 1 and 2, respectively, and 3 μ l of crude antigen extract (5 mg/ml) was loaded into well #3. After electrophoresis, as described, C6 antibody was added to fill trough 1-2 and H9 antibody was loaded to fill trough 2-3.

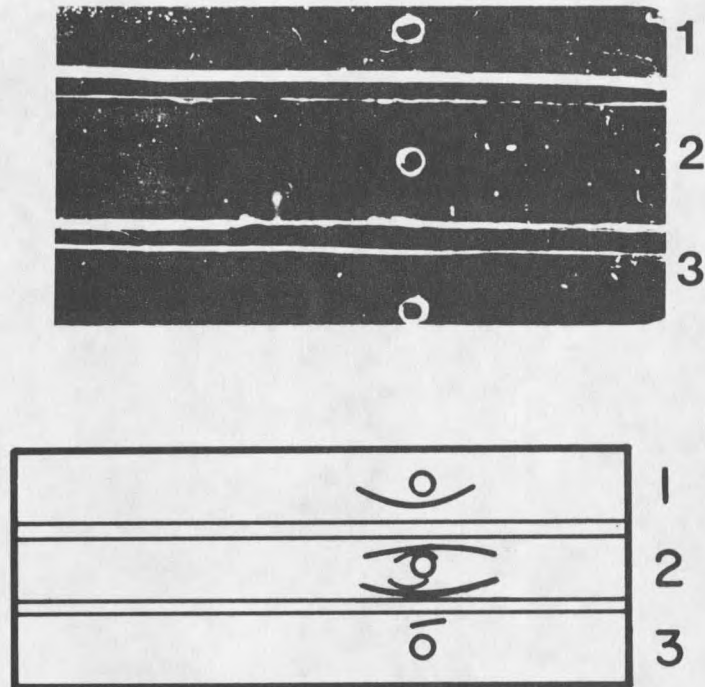


Figure 17. (a) Immunoelectrophoresis (IEM) of HPLC-isolated antigens reacted with antibodies C6 and H9 as previously described. Well #1 contained 3 ul of (5 mg/ml) isolated AgC6; well #2 contained 3 ul of (5 mg/ml) isolated AgH9; well #3 contained 3 ul of (5 mg/ml) crude antigen extract. After electrophoresis both troughs were filled with equal quantities of antibodies C6 + H9. (b) reproduction of the IEM photograph in (a). Bands which did not reproduce for the photograph were visible on the original agarose slide as presented here.

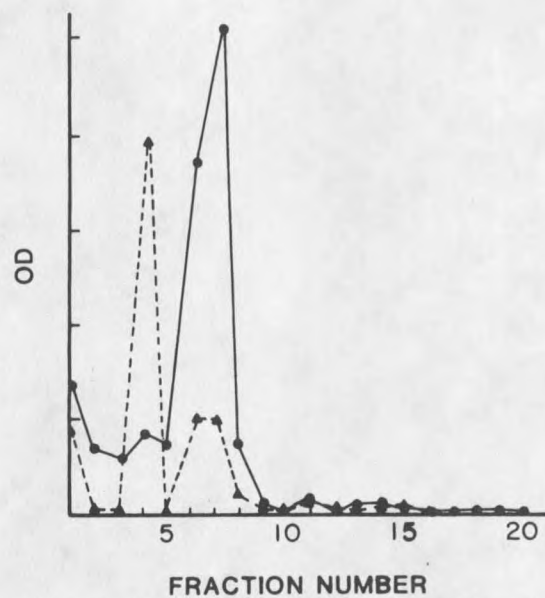


Figure 18. Spectrophotometric readings (at 220 nm) of fraction 1 (●—●) and 2 (—▲—) HPLC-isolated AgC6.

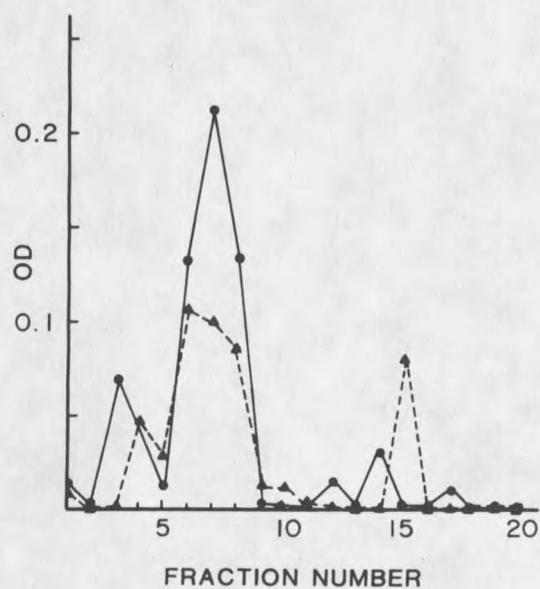
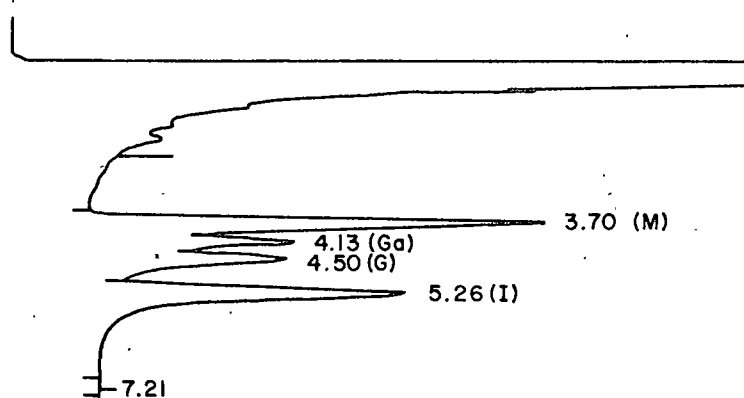


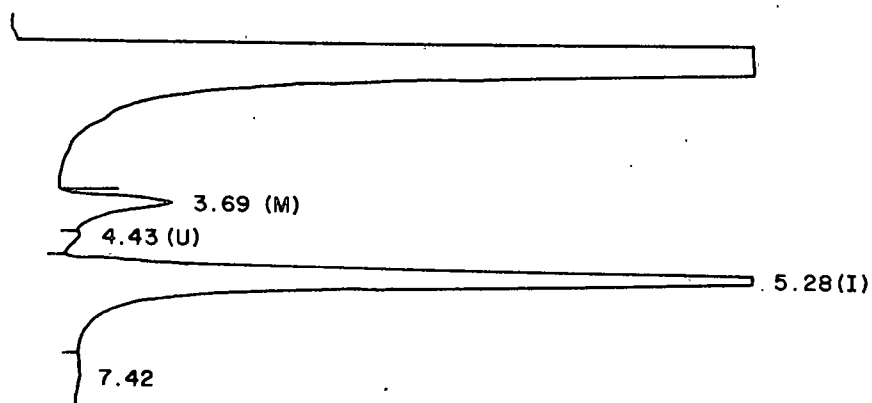
Figure 19. Spectrophotometric readings (at 220 nm) of fraction 1 (●—●) and fraction 2 (—▲—) HPLC-isolated AgH9.

C6 and H9 in Ouchterlony tests. Because the antigens eluted from the column in the same fraction as blue dextran (void volume), the molecular weights of the antigens are probably greater than 10^6 . Molecular weight standards (cytochrome c, MW = 12,500 and phosphorylase b, MW = 97,400) were retained by the column and eluted in the fractions which were predicted by the column manufacturer's specifications. Column-purified antigens were lyophilized and 1.0 mg of AgC6, AgH9, and carbohydrate standards were analyzed as alditol acetates by gas liquid chromatography (GLC) on a Varian 3700 GLC equipped with an ECNSS Gas Chrom Q glass, polar column (Figures 20-22) and on the Varian 3700 equipped with a DB5 nonpolar capillary column (Figure 23, 25, 27, and 29). The DB-5 column has a great resolving capability (greater number of plates) than the ECNSS column. Carbohydrates eluted from the DB5 column in an order different than on the ECNSS column (Figure 20 and 23). Elution peaks from the ECNSS column at 3.69 min (peak M of AgC6, Figure 21) and 3.85 min (peak M AgH9, Figure 22) are equidistant from the inositol internal standard peaks (labeled I at 5.28 and 5.45 min, respectively), a difference of 1.59 and 1.6 min. This compares favorably to the difference in elution times of inositol and mannose standards of 1.56 min (Figure 20). Therefore, peaks at 3.69 min (C6, Figure 21) and 3.85 min (H9, Figure 22) represent mannose. The ENCSS



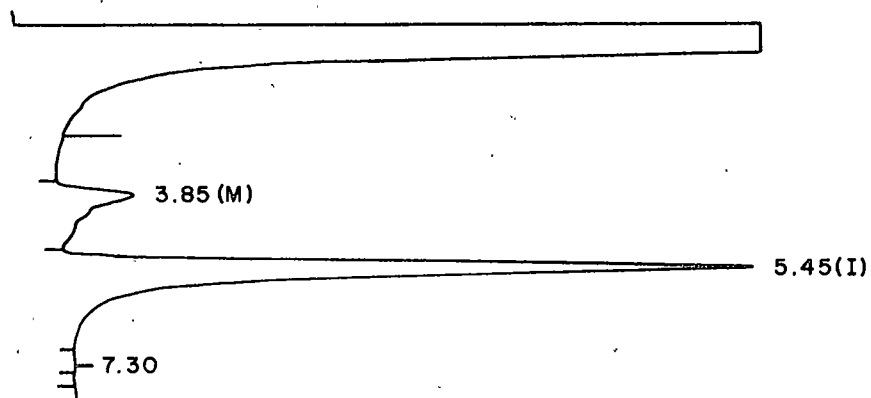
Retention time (min)	% Area
3.70	32.4
4.13	15.63
4.50	19.2
5.26	32.17
7.21	.052

Figure 20. GLC elution profile of acetylated carbohydrate standards obtained from the Varian 3700 equipped with an ECNSS-M Gas Chromo-Q glass column. (M = mannose, Ga = galactose, G = glucose, and I = i-inositol).



Retention time (min)	% Area
3.69	9.12
4.43	1.73
5.28	84.21
7.42	4.93

Figure 21. GLC elution profile of acetylated AgC6 obtained from the Varian 3700 GLC equipped with an ECNSS-M Gas Chromo-Q glass column.



Retention time (min)	% Area
3.85	11.37
5.45	88.42
7.30	.22

Figure 22. GLC elution profile of acetylated AgH9 obtained from the Varian 3700 GLC equipped with an ECNSS-M Gas Chromo-Q glass column.

column and alditol acetates have been previously used to specifically identify cell wall carbohydrates (2, 55, 57). When examining the GLC elution profile of AgH9 by ECNSS GLC analysis, a single peak (I) represents the internal inositol standard (Figure 22). However, several peaks were visible when the inositol standard was run through the DB-5 column (Figure 25). Although the same (DB-5) column was used to run the electron ionization of all samples, (controls, AgC6 and AgH9), the runs were completed on different days. AgC6 and AgH9 (Figures 27 and 29, respectively) were run first and 1 week later the combined standards (Figure 23), the inositol standard (Figure 25), and AgC6 and AgH9 (not shown) were run. In the interim between runs, the column was damaged and 1.0 meter was removed from the terminus which affected the resolving capacity of the column (enough to influence the resolution of the inositol peaks). However, the profiles suggest that 1 additional peak (c), which appears to be similar to inositol in fractionation pattern and elution time, may be present in H9 (comparison of Figures 25 and 29). This peak is not present in elution profiles of AgC6 (Figure 27). This speculation is further supported by results of the hapten inhibition studies. Precipitin bands formed on the control plate between H9 and C6 antibodies and the 5, 2.5 and (to a small extent) 1.0 mg/ml antigen concentrations. Ten percent concentrations

02-MAY-85
INOSITOL MANNOSE & LACTOSE STANDARDS

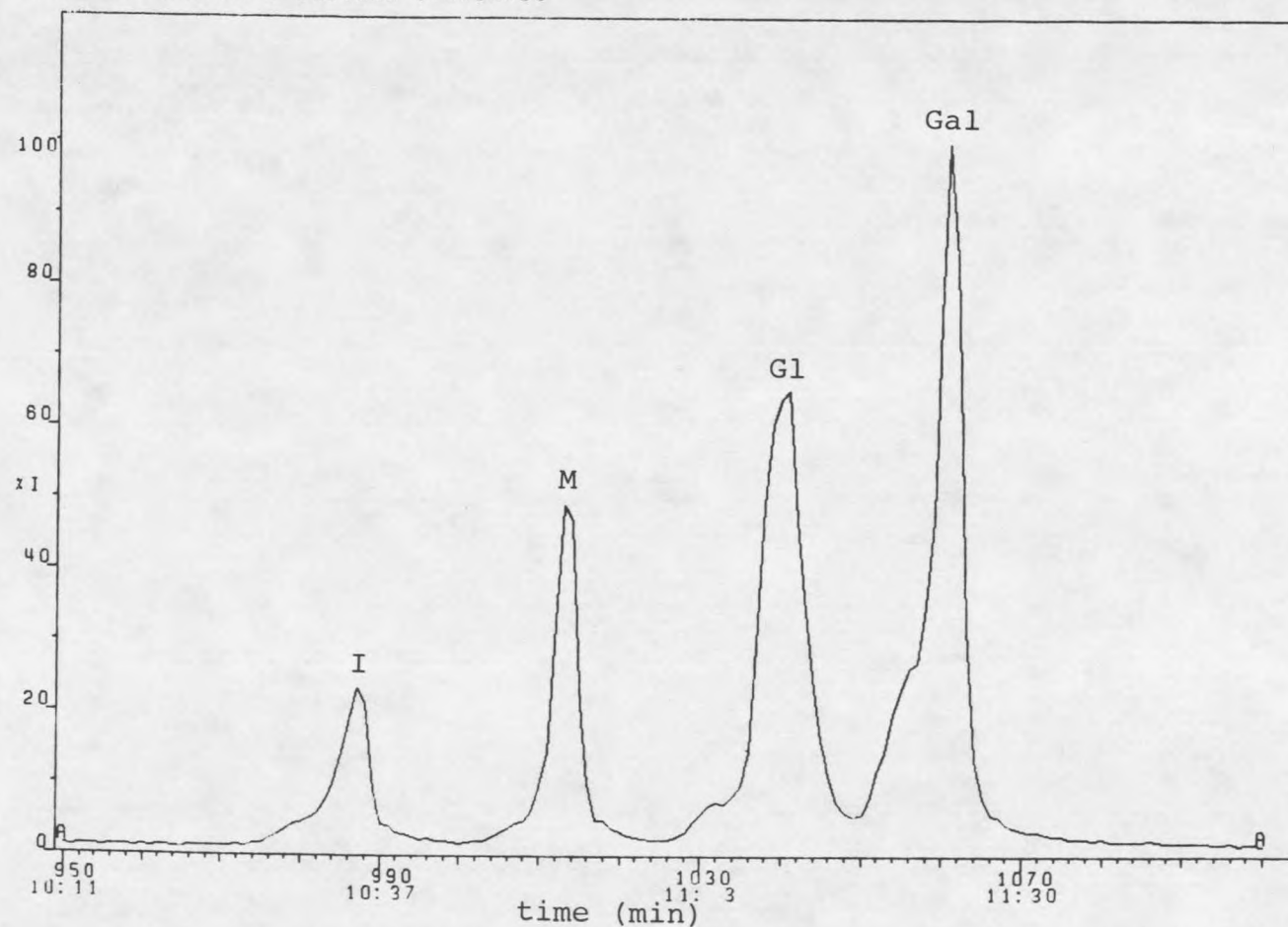


Figure 23. GLC elution profile of acetylated carbohydrate standards on the Varian 3700 GLC and DB-5 column; I=inositol, M=mannose, Gl=glucose, Gal=galactose. Retention times are 10:33 (min:sec), 10:52, 11:11, and 11:24, respectively.

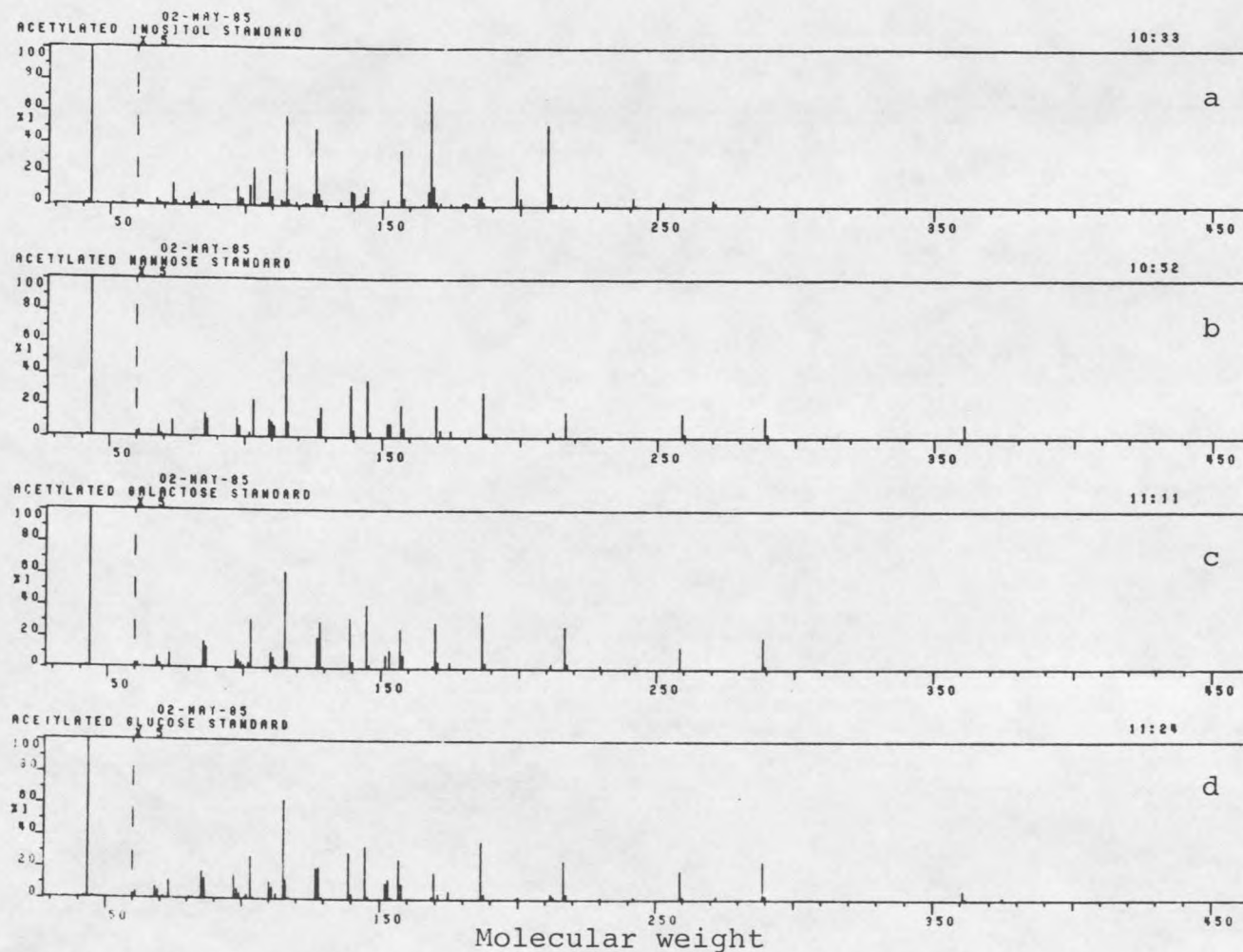


Figure 24. Comparison of electron ionized fractionation patterns and retention times (R.T.) from acetylated carbohydrate standards. a) inositol R.T.=10:33; b) mannose, R.T.=10:52; c) galactose, R.T.=11:11, and d) glucose, R.T.=11:24.

INOSITOL STANDARD 10-MAY-85

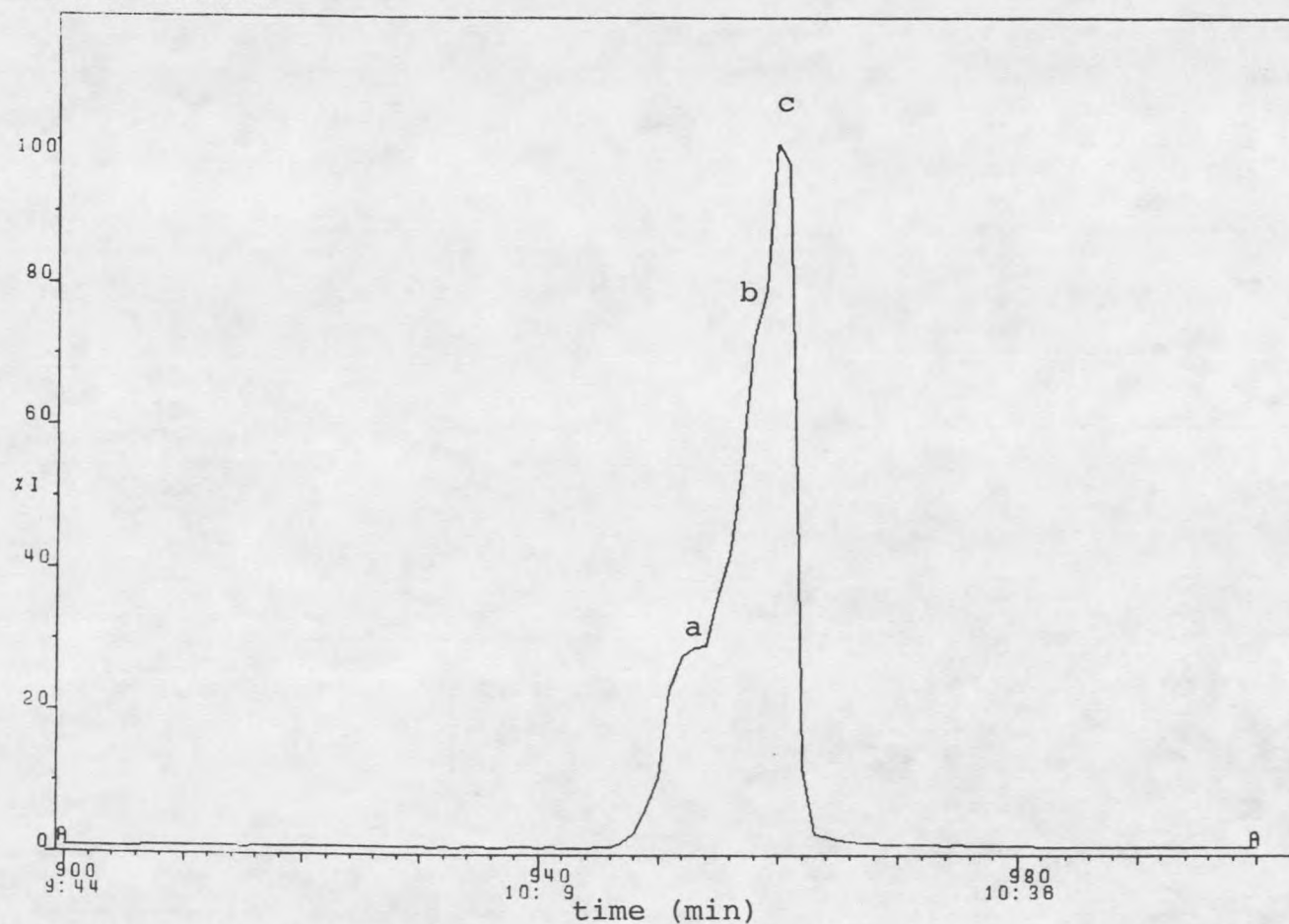


Figure 25. Elution profile of acetylated inositol standard on the Varian GLC with the DB-5 column. Three peaks a, b, and c at R.T.= 10:15, 10:21 and 10:25, respectively, are not well separated.

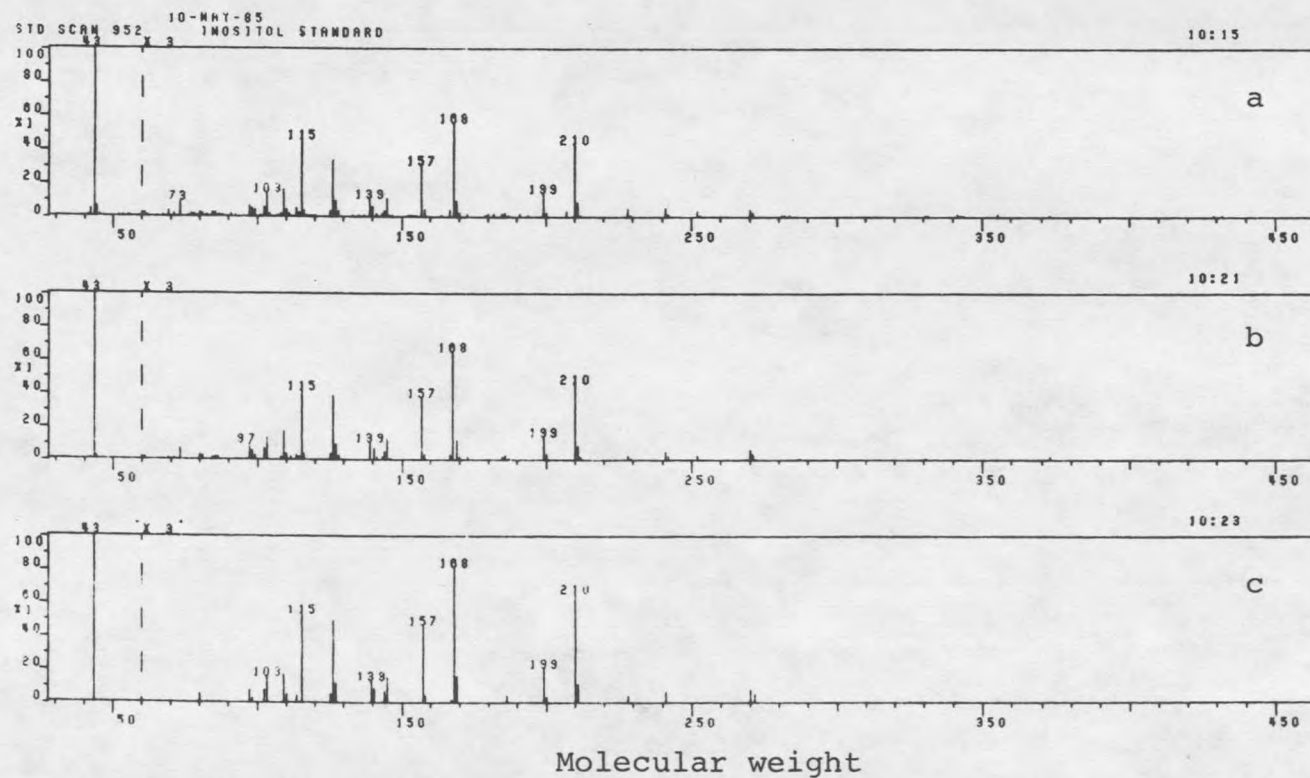


Figure 26. Fractionation pattern of the inositol standard as determined by electron ionization mass spectroscopy. Letters correspond to peaks in Figure 25.

C6.
HPLC

02-MAY-85

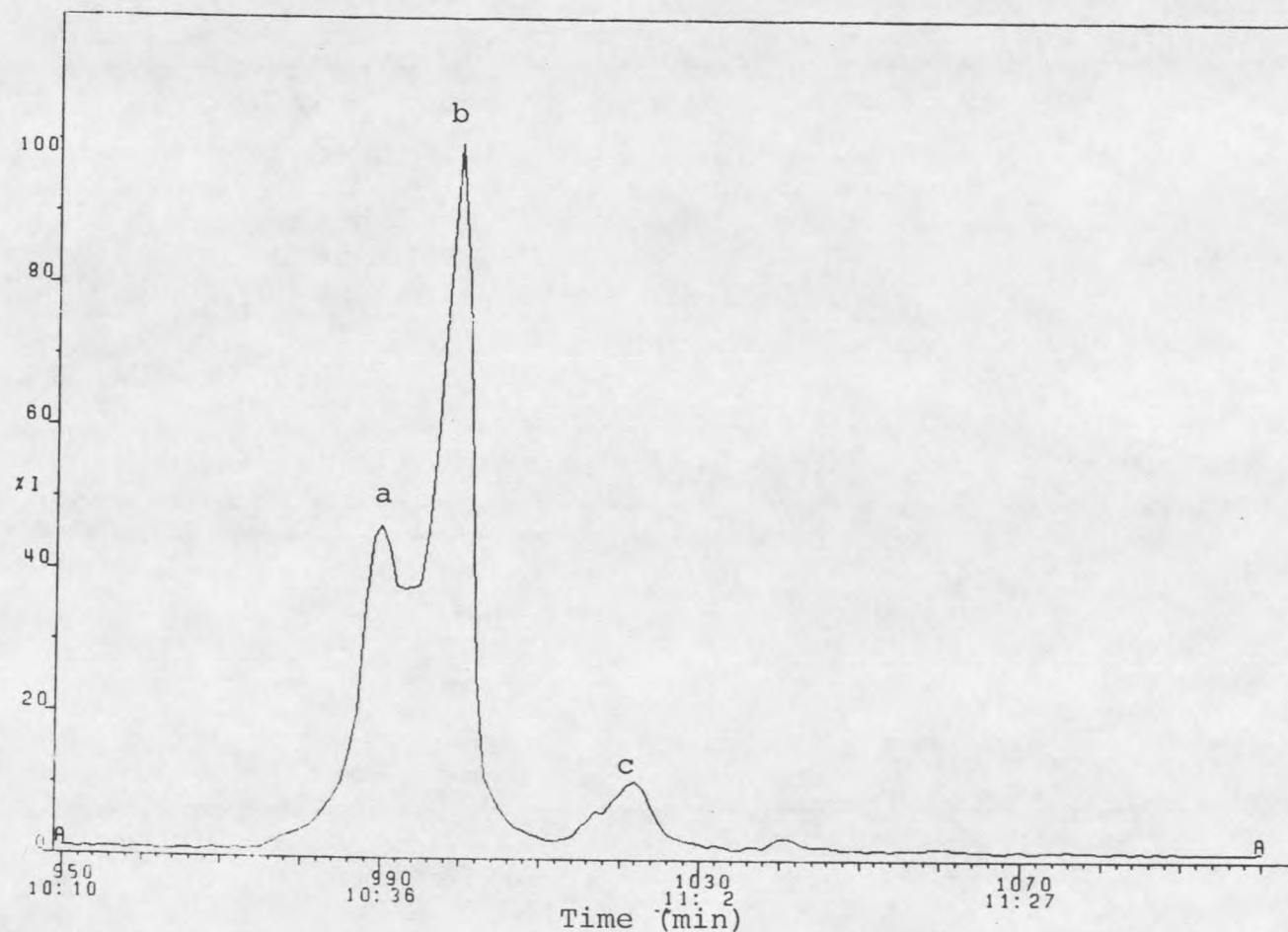


Figure 27. Elution profile of acetylated AgC6 on the Varian GLC (DB-5 column) Peaks a (R.T.=10:33) and b (R.T.=10:40) are internal standard inositol. Peak c) R.T.=10:54) is mannose.

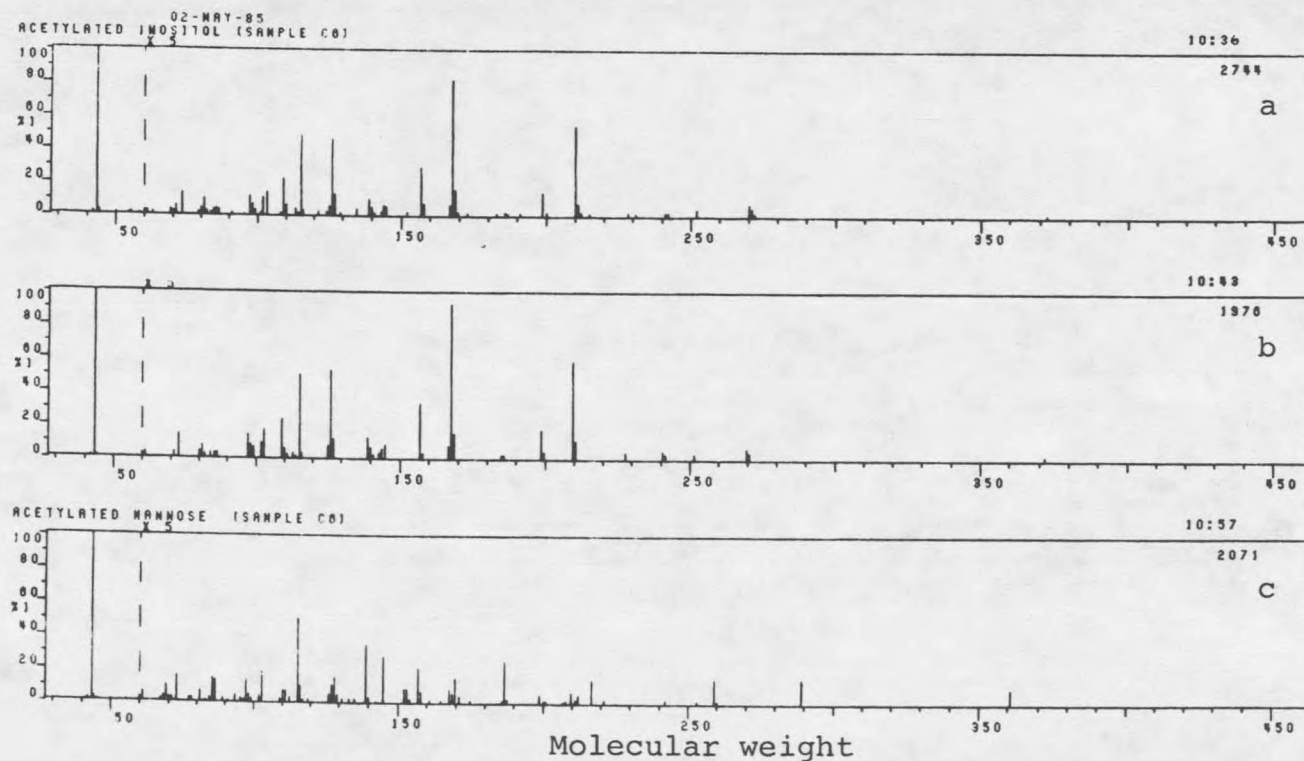


Figure 28. Fractionation pattern of acetylated AgC6 as determined by electron ionization mass spectroscopy. Letters correspond to peaks in Figure 27.

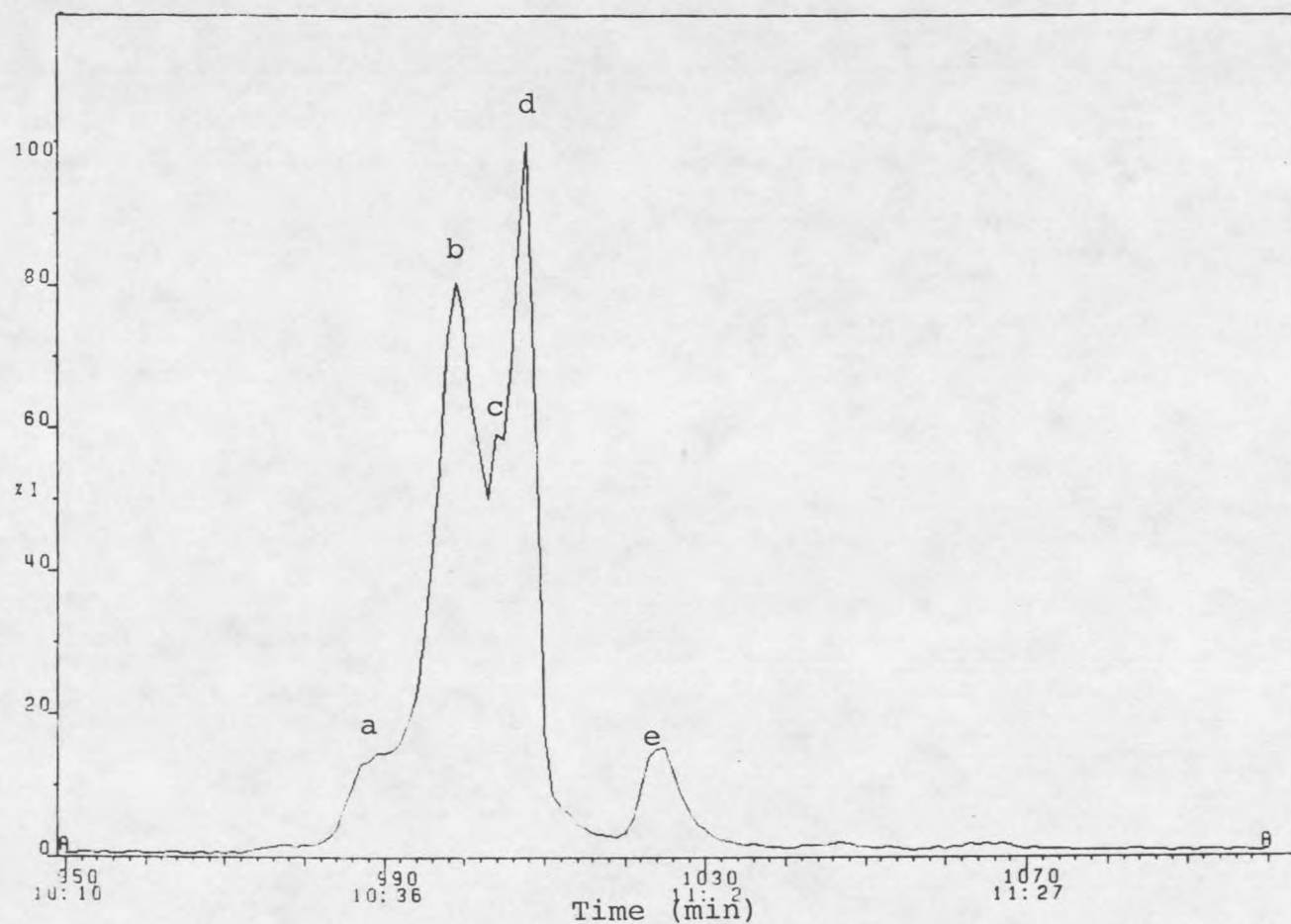
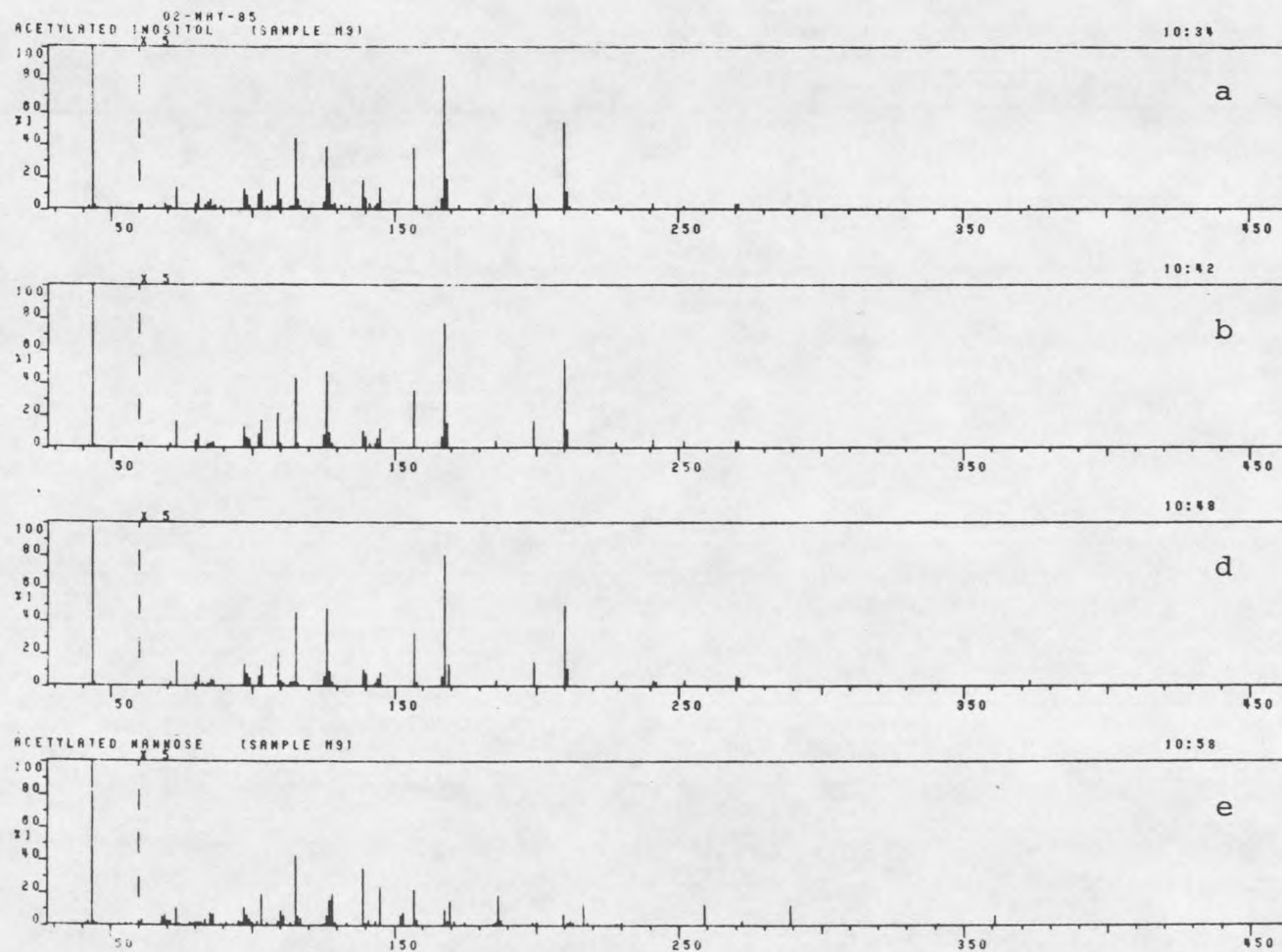


Figure 29. GLC elution profile of acetylated AgH9 on the Varian 3700 (DB5 column). Peaks a, b, and d probably represent the internal inositol standard. Peak c may represent another compound which is "inositol-like". Peak d is mannose. R.T=10:34 (a), 10:40 (b), 10:48 (d), and 10:57 (c).



Molecular weight

Figure 30. Fractionation pattern of acetylated AgH9 as determined by electron ionization mass spectroscopy. Letters correspond to peaks in Figure 29. Peak c could not be differentiated from peaks b or d.

of glucose, rhamnose, arabinose, galactose and trehalose did not interfere with antigen-antibody reactions (precipitate formation) when either H9 or C6 antibody was tested against 5 mg/ml crude antigen extract. However, 10% mannose inhibited precipitate formation when H9 and C6 antibodies were tested. Precipitate formation was inhibited by the presence of i-inositol and meso-inositol only when H9 antibody was tested.

The Varian 3700 column was interfaced directly with a NG7070E-HF double focusing mass spectrometer, and fractionation patterns and peak retention times of the mass spectroscopy data obtained from the electron ionization of standard sugars (glucose, mannose, galactose, and inositol) are shown in Figure 24 and 26. Those of AgC6 and AgH9 are seen in Figures 28 and 30, respectively. The mass spectrometer is capable of ionizing samples with either electrons or chemical degradation (ionized methane, CH_5^+). Both methods yield fractionation patterns which are characteristic for a substance, but the latter method is less destructive and allows a more accurate assessment of the molecular weight of each eluted peak. Electron and chemical ionization fractionation patterns of inositol are shown in Figures 26 and 31, respectively. The molecular weight of the alditol acetate of inositol was determined to be 342 d (Figure 31, inositol = 180 + 6 acetyl groups). The largest

fractionation peak (MW = 273d) represents acetylated inositol minus 1 acetate group (MW = 69d) which was readily ionized by both ionization procedures. The molecular weight of the mannose standard was determined by similar methods from the fractionation pattern shown in Figure 32. The largest fractionation peak was reproducibly 275d, which represents the acetylated 6 carbon structure minus an acetate group or acetic acid group.

Mass spectroscopy revealed that AgC6 and AgH9 contain mannose based on location of peaks, retention time (as previously discussed), chemical fractionation patterns, molecular weight (Figure 34 for AgC6 and Figure 36 for AgH9), and electron ion fractionation patterns (discussed above) of the antigen components as compared to known standards.

The internal standard, meso-inositol, was chosen because inositol has not been reported in cell walls of C. albicans. Possibly as many as 3 peaks are discernible in the inositol standard (Figure 25). Comparing the elution profiles from the GLC studies (as discussed above), fractionation patterns from the mass spectroscopy of inositol standards (Figures 26 and 31), and inositol peaks in AgC6 (Figure 33) and AgH9 (Figure 35) raises the possibility that an additional compound may be present in AgH9 which is not present in AgC6. These results combined

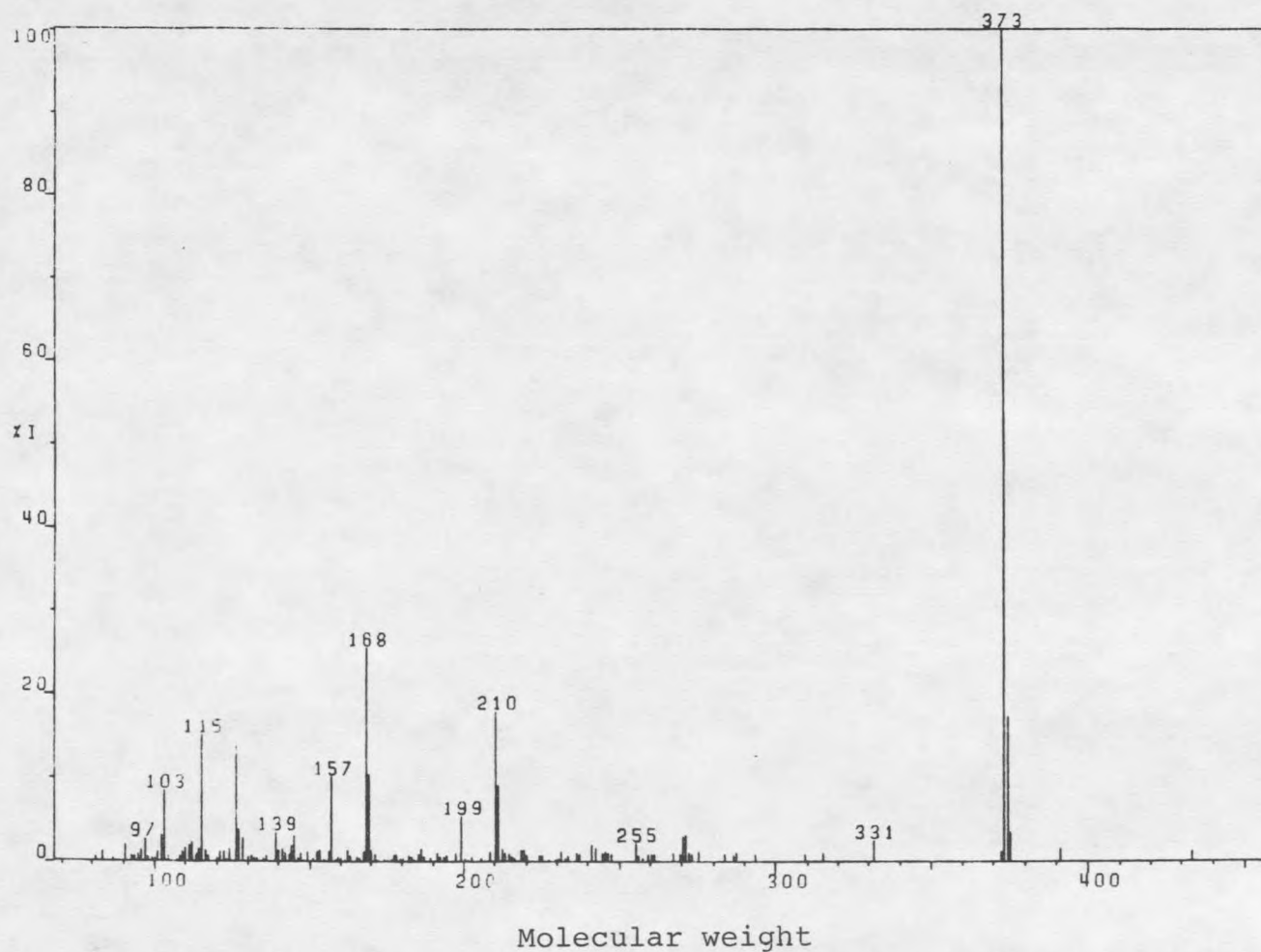


Figure 31. Fractionation pattern of acetylated inositol (standard) as determined by chemical ionization. Peaks represent molecular weights of fragments and their relative proportions.

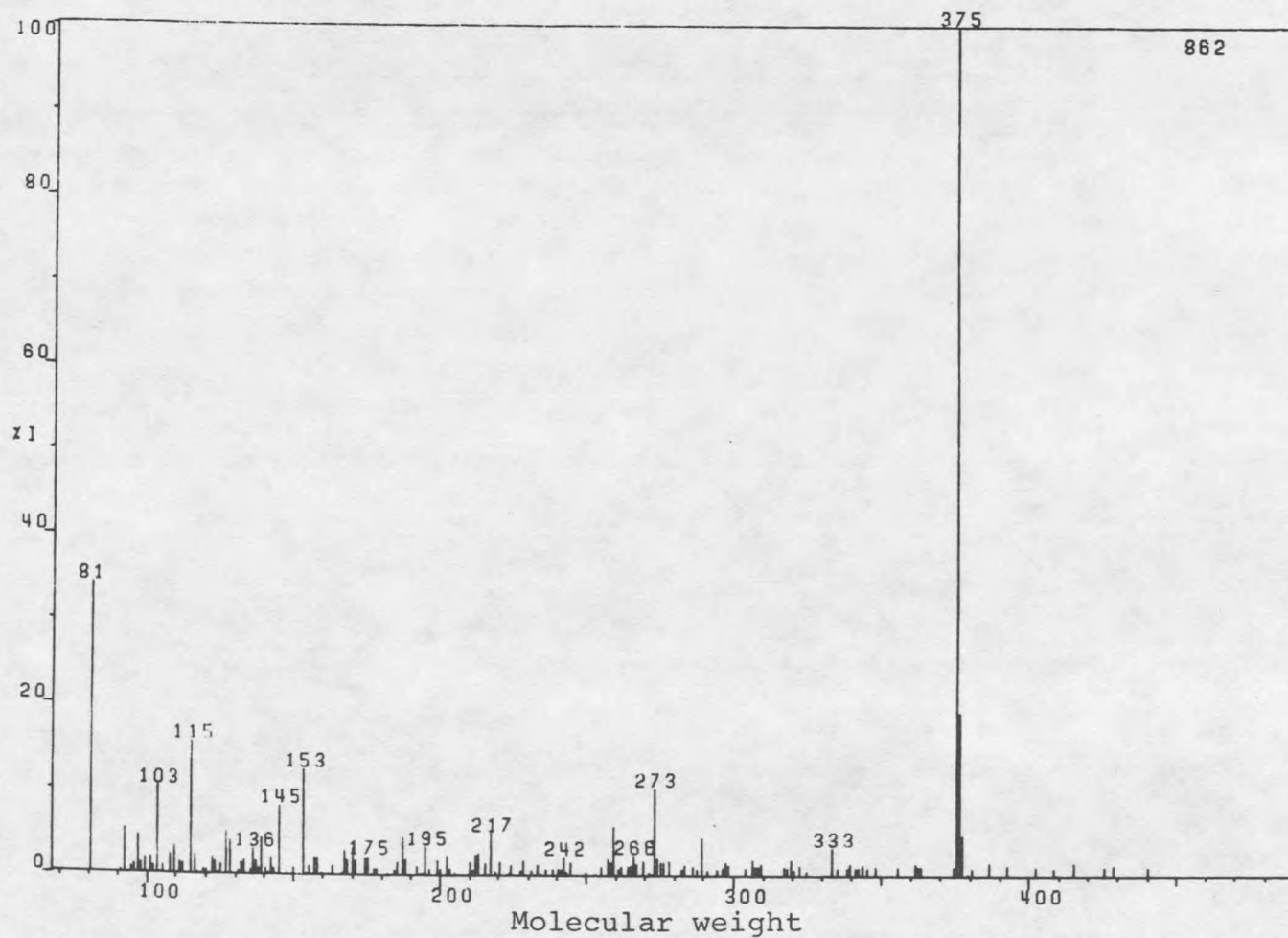


Figure 32. Fractionation pattern of acetylated mannose standard as determined by chemical ionization mass spectroscopy. Peaks represent molecular weights of fragments and their relative proportions.

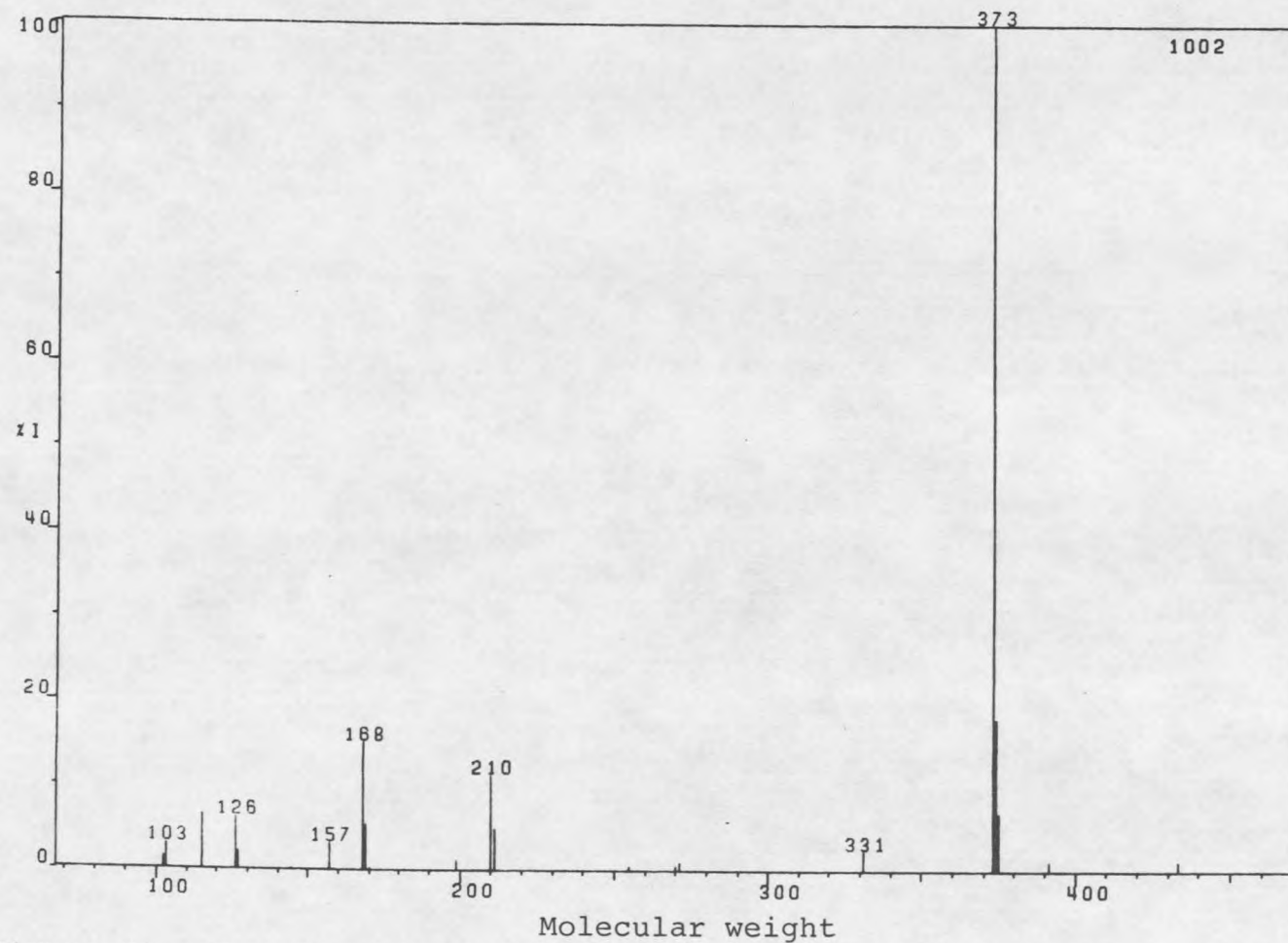


Figure 33. Fractionation pattern and molecular weight determination of acetylated AgC6 inositol (internal standard) component by chemical ionization mass spectroscopy.

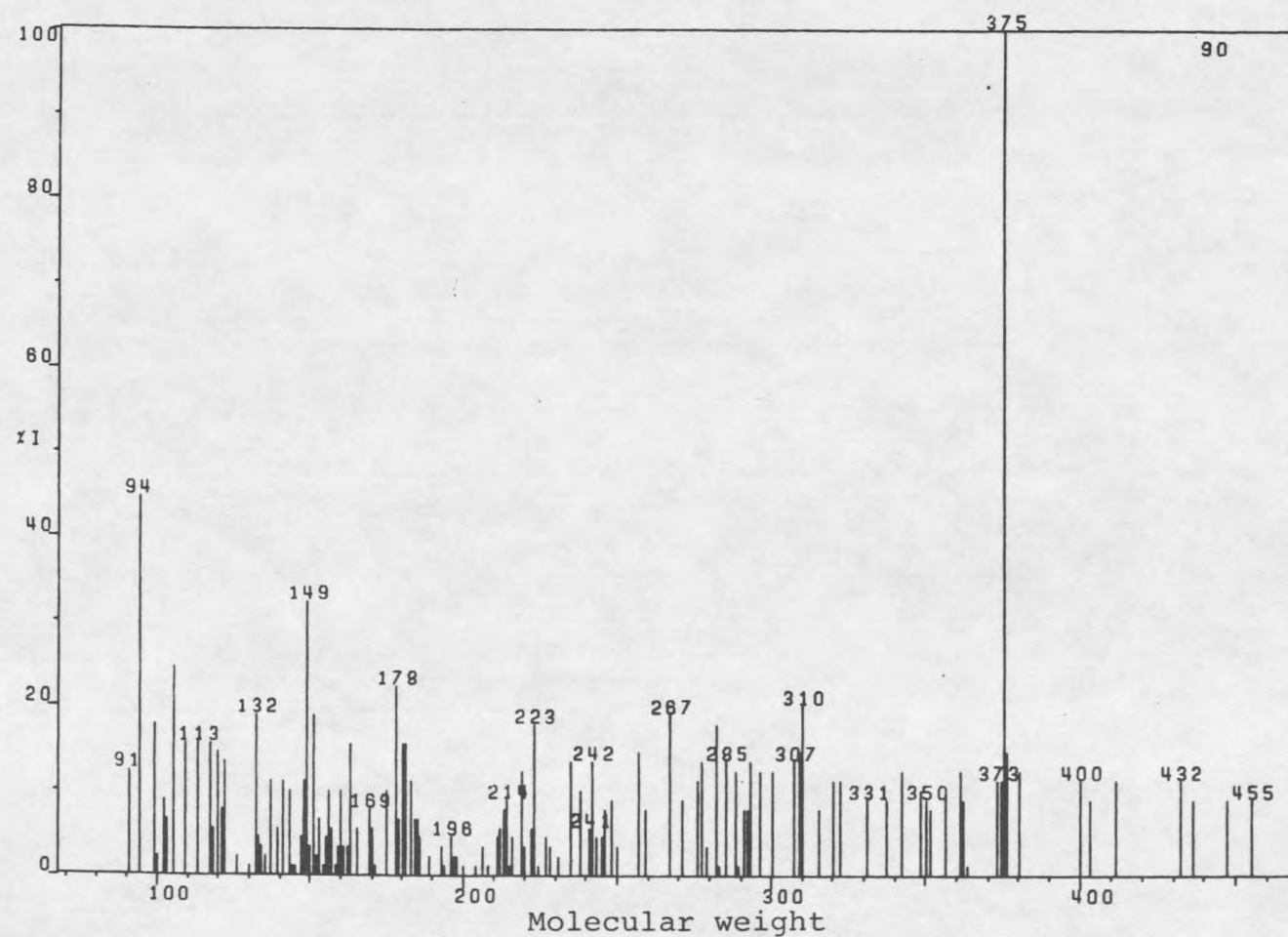


Figure 34. Fractionation pattern and molecular weight determination of the acetylated AgC6 mannose component by chemical ionization mass spectroscopy.

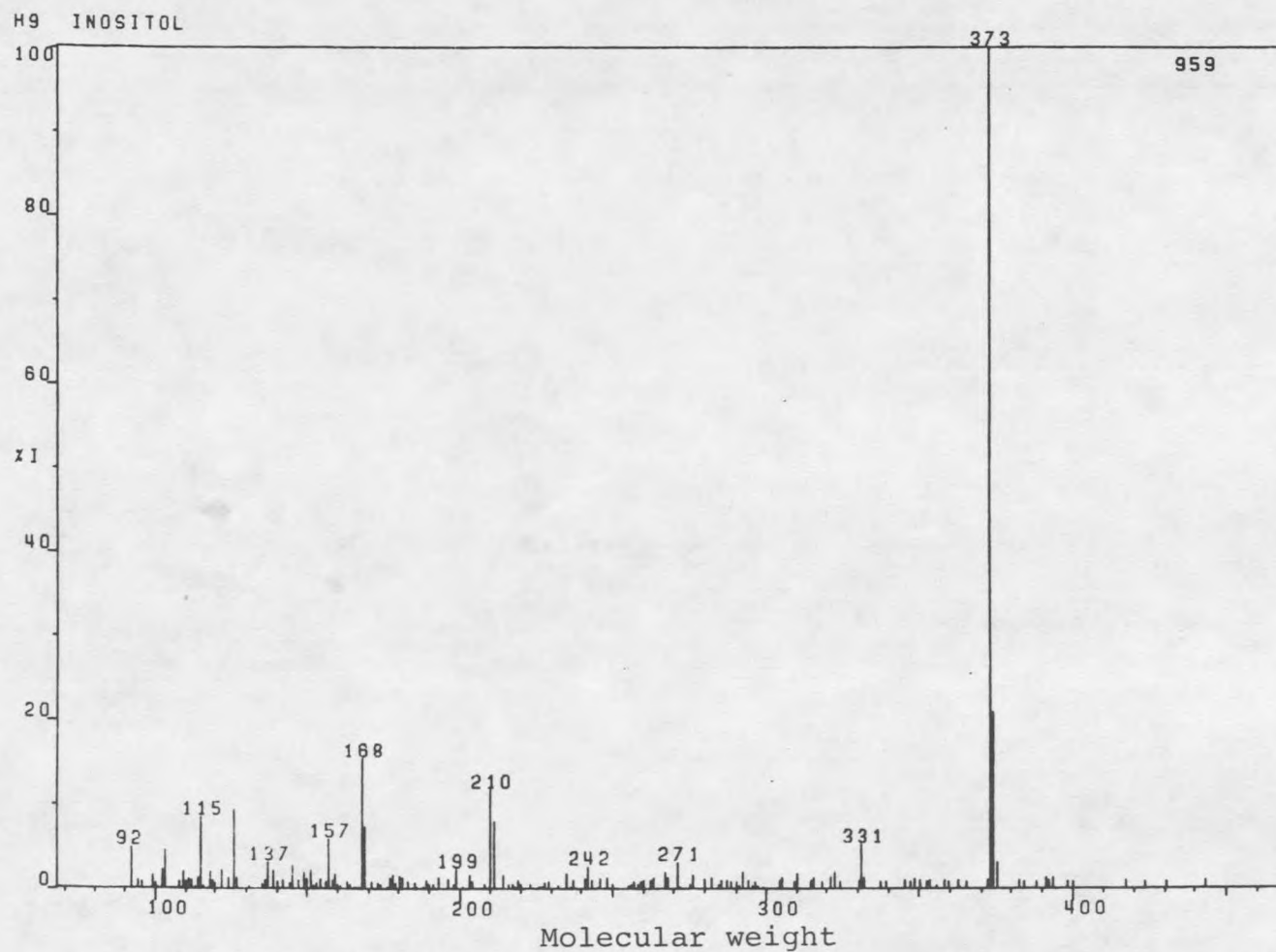


Figure 35. Fractionation pattern and molecular weight of acetylated AgH9 inositol component (probably internal standard) by chemical ionization mass spectroscopy.

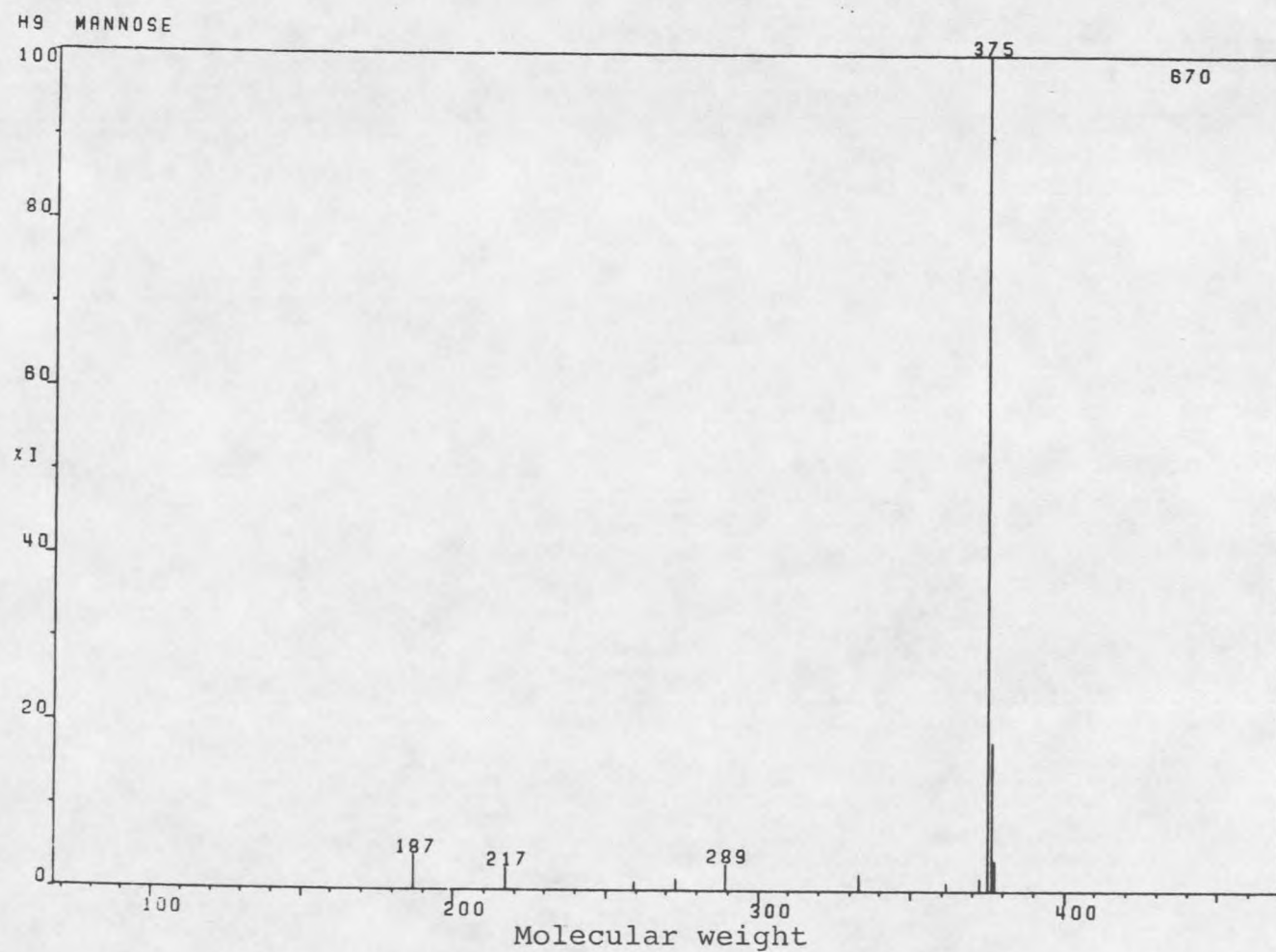


Figure 36. Fractionation pattern and molecular weight determination of acetylated AgH9 mannose component by chemical ionization mass spectroscopy.

with the double overlapping bands of identity which appeared in Ouchterlony tests indicate that AgC6 and AgH9 are closely related compounds viz. both contain mannose which is probably a mannan backbone which could be modified by additional carbohydrate residues. The most likely candidate for the modification based on the elution profiles and hapten inhibition tests is inositol or a closely related compound.

DISCUSSION

In these studies monoclonal antibodies that agglutinate C. albicans strains were isolated from culture filtrates of cloned splenocyte-myeloma hybrid cells. Although 12 hybridomas produced antibody directed against cell wall determinants of C. albicans (as evidenced by the ELISA test), only 3 hybridomas (B6, C6, and H9) yielded antibody which also agglutinated the yeast cells. These agglutinins were characterized as IgM class antibodies, and all were stable to storage at 6-8°C or at -20°C. C6 and B6 were heat-stable while H9 was inactivated by heating for 5 min at 60°C. The characteristics of these agglutinins are similar to those of an agglutinin which appears in mouse sera following immunization with heat-killed C. albicans (173). Bands of identity which formed in Ouchterlony tests (as described above) and agglutinin cross-reactivity of yeast strains tested with C6 and B6 indicated that these 2 antibodies might be reacting with the same surface epitope. Subsequent testing was, therefore, confined to comparisons between C6 and H9.

Antibody H9 was produced and characterized approximately 1 year before C6. Consequently, initial agglutination, indirect fluorescent antibody, and media comparison studies were confined to the use of this agglutinin. The immunodeterminant specific for H9

appeared to vary in concentration on individual strains and serotypes of C. albicans and was detected on C. tropicalis and T. glabrata (Table 2). The antibody did not agglutinate other Candida sp. The association of the (H9-specific) antigen with the cell wall of T. glabrata appeared to differ from its association with the cell wall of C. albicans in that 1 M NaCl treatment did not remove the antigen from cells of T. glabrata. The absence of agglutinin reactivity in some strains of yeast was apparently due to the lack of primary binding of antibody rather than inhibition of agglutination because: 1) S. cerevisiae neither agglutinated with antibody nor absorbed the agglutinin from solution; 2) weakly reactive strains of C. albicans effected only small reductions of agglutinin titer following absorptions (Table 6); 3) a weakly reactive strain, C. albicans 394, which was mixed with the agglutinin and then washed with saline was weakly reactive with fluoresceinated anti-mouse immunoglobulin.

Growth curve analyses revealed 3 patterns of H9 reactivity: 1) agglutinin reactivity in several organisms reached its maximum (4+) by 5 h and reactivity after 8 h was media dependent (Figure 2a), 2) agglutinin reactivity peaked (4+) only briefly at 6-8 h, prior to and after which time both agglutination and autoagglutination were transient and media dependent (Figure 2b), and 3)

agglutinin reactivity in several organisms steadily increased from 2-4+ during early log phase reaching 4+ reactivity by 6 h and remained 4+ throughout the remainder of the experiment (Figure 2c). Furthermore, in media comparison studies, cells grown in either complex or chemically defined broth media tended to react with the agglutinin more strongly than cells grown on the surface of solidified media (Table 1). These results indicate that the expression of at least 1 cell wall determinant is dependent upon species, strain, growth phase, environment and nutrition.

Fluorescent antibody studies of H9-specific surface antigen expression on C. albicans 9938 correlated well with H9 agglutinin reactivity observed at 5 h incubation (see Results and Figure 2c). The 24 h fluorescence of C. albicans 9938 cells appeared weaker than the 5 h fluorescence while slide agglutinin reactivity remained strong (4+). This may reflect the increased sensitivity of fluorescent antibody tests over agglutination tests. During the most reactive growth phase (5 h) only 50% of the C. albicans 9938 cells appeared to react with the monoclonal antibody which indicates either that some cells are suppressed from antigen expression or that cells alternate between expression and non-expression and shed antigen. In support of the latter hypothesis, antigen is readily solubilized by relatively mild (1 M NaCl)

extraction procedures, and soluble antigen is detectable in culture filtrates. These ideas would be most appropriately tested by studying cells in synchrony, a feat we have been unable to maintain in C. albicans for more than 1 generation. However, studies in which we used immunoelectron microscopy to study the location of surface antigens lend support to the latter hypothesis.

Many previous investigators who used absorbed polyclonal antisera (42, 48, 49, 50, 51, 140, 151, 155, 156, 157, 158, 162) have examined cell surface determinants and cytoplasmic antigens mainly for taxonomic purposes. Such studies may presuppose a static antigen display on the surface of C. albicans. In accordance with other studies of surface antigens of yeast (50, 51), we found that C. albicans shared antigens also expressed by C. tropicalis and T. glabrata. Furthermore, although the quantity of antigen expressed on the cell surface was dependent on growth phase and nutritional factors, yeast species which did not react with the agglutinin seemed incapable of antigen production under any conditions.

Results of our studies raise questions about the validity of arguments as basic as whether C. albicans exhibits antigenic homogeneity or heterogeneity (42, 48, 162). Our observations suggest that classification of C. albicans into 2 or 3 serological groups (42, 50, 104, 162) is oversimplified in that antigenic expression is a

dynamic rather than a static process and is influenced by strain variation, growth phase and environmental conditions. Furthermore, serotyping of the strains of C. albicans which we used in agglutination studies does not correspond to the antigenic groups designated by H9 agglutinin reactivity (Table 1). For example, using only 2 types of media, we observed 3 patterns of agglutinin reactivity during growth of various strains of C. albicans (Figure 2). Disagreements regarding serological classification of yeasts (42, 50, 104, 162) may reflect problems which stem from varied methods of antisera production, the varied nature of polyclonal antibody responses (and the different immunization schedules used to produce them) as well as the different growth environments to which test organisms are exposed.

Monoclonal antibody C6 was isolated 1 year later than H9, and characterized as a second IgM agglutinin. Ouchterlony double diffusion tests with heated 1 M NaCl antigen extract and reactivity with various yeast strains (Table 3) indicated that C6 and H9 antibodies are specific for different epitopes on yeast surfaces. All strains of C. albicans tested were reactive with H9 and C6 agglutinin, (130 and 52 strains, respectively, reported in part in Tables 2 and 3). However, the antibodies differed in their degree of reactivity among strains of C. albicans, and they appeared to react with different

antigenic determinants as evidenced both by the bands of nonidentity which formed in Ouchterlony double diffusion tests and by differences in the agglutinin reactivity among yeast strains viz. when 3 strains of T. glabrata were tested with both agglutinins, H9 antibody was strongly reactive with each of the strains while C6 was nonreactive. Both monoclonal antibodies agglutinated C. tropicalis (although to different degrees) but no other strains or species of yeast tested were reactive with either agglutinin. C. albicans blastoconidia autoagglutinated during early log phase (5-7 h, Table 4 and Figure 2).

Immunoelectron transmission microscopy allowed us to locate and follow the expression of surface antigens on C. albicans during growth. This method is refined in comparison to the agglutination techniques first used to detect the antigens, and semiquantitative assessments could be made with this technique. I found that experiments in which colloidal gold was used to label determinants were superior to those in which ferritin was used as a label because the larger colloidal gold particles were visible at low magnifications (5000-10,000 x), and lower magnifications allowed examination of an entire cell periphery to enumerate particles bound/cell. Use of ferritin labeling would have particularly hindered examination of germinating cells due to the large size of

a mother cell with attached germ tube. Background electron imaging (BEI), which superimposes an SEM and BEI image, would be the preferred method of quantifying surface determinants for 2 reasons: 1) SEM yields a three dimensional view of the cell surface and allows one to observe label on entire surfaces rather than just the surface periphery visualized by TEM and 2) BEI specifically detects gold particles on a surface by atomic number and, thereby differentiates label from debris. Discerning label from debris was inconsequential in TEM examinations of colloidal gold-coated cells, but was a significant problem with SEM evaluations when the ELADA technique was done.

Antigenic determinants AgC6 and AgH9 appeared to be associated with a flocculent or fibrillar cell wall coating similar to one which may be involved in adherence of C. albicans to host cells (161). In our studies, a direct correlation existed between the expression of this layer and the presence of the colloidal gold on the surface of the yeast cells tested. Cells which did not possess this layer, including C. parapsilosis, did not bind colloidal gold more than negative control cells.

Previous studies have shown the presence of a fibrillar or flocculent layer as one of the outer 5 (to 8) cell wall layers of C. albicans (34, 123). In one study, this layer was not detected when cells were grown in

liquid medium and expression of it was dependent on the growth medium used. The layer was not present on surfaces of bud cells although it was expressed on surfaces of mother cells, and is reportedly composed of thin filaments which stain with polysaccharide detection techniques (123). In our work, which was done exclusively in liquid medium, expression of neither the antigenic determinants nor the flocculent layer occurred on the surface of young bud scars or during early stages of bud formation but as the bud scar matured, the flocculent layer was expressed and, therefore, colloidal gold was bound at the antigenic site.

Yeast examined by TEM in early stages of growth (4-6 h) were not in synchrony in that cell size and amount of immunolabel on cell surfaces varied from negative to moderate. Autoagglutination, which interfered with the interpretation of cell surface antigen expression in agglutination studies, did not mask or interfere with detection by immunoelectron microscopy nor did cells in early log phase bind the colloidal gold-labeled antibody nonspecifically. Neither the thickness of the flocculent layer nor the size of the blastoconidia correlated with the amount of gold label. In fact, in some cases, the smaller cell populations were more heavily labeled with gold than larger cells. However, determinations of the amount of surface label present were made only after

observing a large number of cells from all time periods in at least duplicate experiments because, (due to the transient nature of antigen expression within the population) at any time period when cells were treated with agglutinin, a rare negative cell could inevitably be found among the majority of positive cells with arduous searching. Conversely, one could also find a rare positive cell among cells with no bound label, with the exception of the negative controls which did not express colloidal gold on their surfaces.

Most agglutination reactions correlated well with the amount of cell surface label observed by TEM. Minor discrepancies were confined predominantly to organisms 9938 and 105 when reacted with H9 in late stationary phase when denuding of the surface layer which contained the antigen was evident (Table 7). Perhaps these (multivalent) IgM monoclonal agglutinins have exceptionally strong affinity for their respective antigens so that even when cells shed surface antigens, C6 and H9 were still capable of agglutinating the yeast cells. This inference is supported by our observations that antigen-monoclonal antibody complexes cannot be dissociated by treatment with 4M NaCl or low pH (glycine-HCl buffer pH 2.2).

Much emphasis has been placed upon defining and understanding dimorphicism of C. albicans because the mold

form is historically associated with the pathogenic form. Not as much information has been gathered on the mechanisms of morphogenesis. During examination of Candida in ELADA and BEI experiments, we noted that germinating cells often appeared collapsed while nongerminating control cells which were suppressed from germ tube formation (yet subjected to identical EM preparatory treatment) were not. Occasional TEM photomicrographs also indicated that cells giving rise to germ tubes tended to collapse (see Figure 9 and 10). This observation seemed to support a novel model of germ tube formation (46) which proposes, based on morphological and ultrastructural evidence, that germination is accomplished by the extrusion of mother cell cytoplasmic contents into the developing germ tube which is accompanied by extensive vacuolation within the mother cell. Vacuolation is a necessary event because C. albicans exhibits linear growth kinetics during germination (46) while the filamentous moulds show exponential growth kinetics (160). Our results indicate that as the germ tube increases in length, the highly vacuolated mother cell has a tendency to collapse. Although one may assume that collapse occurs consequent to fixative steps or dehydration in preparation for microscopy, the apparent flexibility of the yeast cell wall (Figure 10b) would not preclude collapse in the native state unless turgor pressure were maintained during

the extrusion of the cytoplasm. Regardless, the fixative procedures probably do not cause collapse which is without physiological basis because nongerminating cells do not collapse under the same treatment.

After observing many examples of TEM photomicrographs, we have concluded that germinating cells do consistently appear more vacuolated than nongerminating cells. But in addition to vacuolation, cell wall changes may contribute to vulnerability of the cell to collapse. As previously mentioned, Herman and Soll (58) have recently reported that the initial cell size and volume of the C. albicans mother cell prior to either bud or germ tube formation is the same and the growth rates of the 2 cell types are comparable, yet the cell volume of the bud is 27% greater at any given time point than the germ tube volume. This would indicate either that the kinetics of growth of the budding cell are not linear or that cell wall changes occur by different mechanisms in the 2 populations. Qualitative differences between the composition of mycelial and yeast cell walls have been reported (10, 23). According to both groups, chitin is found in a significantly higher concentration in mycelial phase cells than in yeast phase cells which would suggest that the former cells might expectedly be more rigid. However, my work would seem to indicate that this increased rigidity is not sufficient to compensate for the

cytoplasmic changes which are concomitant with germination. Furthermore, Gow and Gooday (46) suggest in their paper that cells allowed to germinate in liquid medium regenerate their cytoplasmic contents by 3 h while those in solid medium regenerate their cytoplasm by 4 h as determined by phase microscopy. I observed vacuolation in the majority of cells at 8 h. At 20 h growth, those mother cells which could be distinguished in the masses of hyphae appear to have collapsed which indicates either that cell wall differences still exist despite the regeneration of cytoplasm or that cytoplasm is not regenerated. The differences observed also may be due to differences in growth medium used in the study.

In addition to studying the physical mechanisms responsible for morphogenesis, more information is needed to define host-Candida interactions and the role that dimorphism plays in virulence. We used antibodies C6 and H9 to study antigen expression during germ tube formation and found that hyphal forms also express antigens dynamically, a finding that may be integral to understanding pathogenic mechanisms. Antigen which reacts with H9 antibody is expressed transiently during germination while antigen specific for C6 antibody remains invariant. Characterizing the expression of surface antigens could aid in the understanding of Candida-host relationships and explain the confusion regarding the

protective role of antibody in disease. Antigens which are invariant may be important for host recognition and defense whereas antigens which are dynamically expressed could represent immune evasive mechanisms of Candida and may explain the apparent futility of using antibody as a prognostic indicator in systemic disease.

Methods to semi-quantify surface antigens (i.e. 1-4+ bound colloidal-gold particles) were chosen by convention and for convenience. We encountered no problems or discrepancies when this method was used to enumerate gold particles on the surface of nongerminating cells. Except for occasional patches where the surface antigen appeared to be denuding from nongerminating cells, gold label was uniformly distributed on cell surfaces. However, this enumeration system was not as straightforward when applied to germinating cells. Germ tubes were often more heavily labeled at the apices (where the flocculent layer consistently appeared to be most dense) than along the filament, particularly in younger cells. However, we cannot rule out the possibility that in some cases tangential cuts through the flocculent outer layer gave a deceptive appearance of heavier labeling. Photographs of cross sections of hyphae, which are often more numerous in the electron micrographs than longitudinal sections (arrow in Figure 7c), established that labeling occurred also along the filament.

In agreement with Schweritz (137), we found that the flocculent outer layer, viz. the layer which contains both C6 and H9-specific antigens, is continuous from the mother cell to the germ tube and hyphae. This layer appears be thinner along the filament (Figure 6c-f). Although the flocculent layer was present on mother cells at 1, 2, and 4 h, no gold was detected in cells treated with H9 antibody. The presence of the flocculent layer, therefore, does not guarantee antigen expression. Both the flocculent layer and AgC6 are expressed continuously on mother cells and filaments throughout the study. I have shown that yeast cells shed the outermost surface antigens during growth, filaments seem to retain the thin outer flocculent layer throughout at least the first 20 h.

According to McGinnis (94), the base of a pseudomycelium is constricted but the base of a germ tube is not. This characteristic is used as a basis to distinguish these 2 morphological entities. Before each batch of germinating yeast cells was prepared by immunolabeling, I performed cell counts (as described) to determine % germination, differentiating between germ tubes and pseudohyphae on the basis of constrictions. In a series of 3 experiments I recorded the following percentages: experiment 1, germ tubes 58%, pseudohyphae 42%; experiment 2, germ tubes 78%, pseudohyphae 21%; and experiment 3, germ tubes 91%, pseudohyphae 6%. The

longstanding questions of the ontogeny and significance of differentiating pseudomycelium from germ tubes is not one that I wish to address, but I do note that although differentiation between germ tubes and pseudohyphae in electron micrographs is not always reliable because the third dimension is lost, antigenically none of the filaments at any time period in any experiment differed significantly. Therefore, despite reputed differences in ontogeny of true and pseudohyphae (56, 61, 94, 107) both cell types may express antigens in a similar manner. This is demonstrated in Figures 7d and 7e where 7d is constricted and is, therefore, a pseudohyphae while 7e is definitely a germ tube.

Several attempts have been made to develop schemes for the differentiation of strains of C. albicans to determine sources of infection and pathogenic potential (95, 107, 122, 168). These methods are laborious and expensive. Monoclonal antibodies which are directed at surface antigens of C. albicans may serve as useful tools to differentiate yeast strains for epidemiological purposes.

Hybridoma antibodies directed against candidal cell surface determinants represent a potentially powerful tool for gaining a better understanding of fungal-host interactions. Using them, antigens expressed on the surface of the fungal cells, which should be critical in

host recognition of the parasite and in candidal pathogenesis, may be defined. Diagnostic tests which detect serum antibody have, to date, been useless in diagnosing candidiasis. Tests developed from monoclonal antibodies, like the immunospot test we describe, may be valuable in detecting antigens peculiar to the pathogenic candidal form if they are shed into sera of infected individuals.

Variation and shedding of cell surface layers may have important implications in the pathogenesis of C. albicans and in host-parasite interactions. Antibodies directed against cell surface determinants that are readily shed would be one method by which Candida could evade the host immune response and establish infection, particularly when shedding of the surface antigens exposes a moiety responsible for mediating adherence or when these antigens adsorb protective antibody from serum. Based on studies of bacterial adherence and its implied role in pathogenicity (109), a correlation between in vitro adherence and pathogenic potential of C. albicans seems probable and, in fact, in vitro and in vivo adherence as a pathogenic mechanism in candidiasis has been investigated (78, 93, 135, 141, 144, 161). As alluded to previously, when chitin or its derivatives are removed or blocked on the cell surface of C. albicans, adherence is inhibited (78). Adherence to acrylic surfaces is substantially

enhanced by the presence of a preformed outer surface layer which is synthesized in response to the carbohydrate concentration of the growth medium and is also growth-phase dependent (93). My in vitro reports of antigenic variability appear to correlate with reports of in vivo ultrastructural modifications of the cell wall coat of C. albicans during adherence of the organism to buccal epithelium. Adhering yeast cells develop a fibrillar layer which is composed of polysaccharidic granules that may be responsible for attachment (161). Like us, Tronchin (161) reported that the cell wall coat is shed during growth (Figure 4e). Cassone (21) reported that mannan is located throughout all layers of the cell wall, while Poulain (123) stated that mannan and glucan are confined to layers 1-4. Although he could not rule out the existence of chitin in upper cell wall layers, Poulain reported that the majority of chitin exists in layers 5 and 7, and that with aging, layers 1 and 3 disappear, which may correlate with the mannan antigenemia reported in candidiasis.

Although my observations have been confined to cells grown in vitro, to find profound surface changes occurring during pathogenesis of candidiasis would not be surprising. In fact, this has been recently suggested by others using conventional antisera in their studies (124, 125).

Cell wall analyses of C. albicans have, to date, been relatively crude. The rudimentary attempts to identify the composition of cell wall AgC6 and AgH9 have established a basis for the cross-reactivity observed in Ouchterlony tests. Both antigens contain mannose, presumably as mannan. This is logical because mannans and glucans are major components of the cell wall of C. albicans (21, 51, 123, 178, 179) and likely to be a component recognized by the immune system. Indeed, mannan complexes are reportedly antigenic and are responsible for the major humoral antibody responses in humans (61). Inositol is found as a component of cell membranes in the yeast, but due to our preparatory and isolation methods, the presence of inositol (as a membrane contaminant) is unlikely. A second moiety which may be present in AgH9 and resembles inositol in GLC-mass spectrophotometry would explain the overlapping bands of nonidentity seen in Ouchterlony tests and the differences in specificity of H9 and C6 antibodies as determined by immunoelectron microscopy and agglutination data. Minor modifications of carbohydrates in the cell wall of yeasts have been reported although no functional basis for these modifications is presently known. For instance, chitin (beta 1-4 linked N-acetyl-d-glucosamine) and chitosan, an acetylated derivative of chitin, are present in C. albicans cell walls possibly as a link between mannan and

side chain protein moieties (118). Others report that chitin is a cell wall component concentrated in bud scars and found within the glucan network (18, 61, 128).

Nevertheless, I examined the possibility that N-acetylglucosamine was a constituent of AgC6 and AgH9 by GLC-mass spectroscopy and found that the amino sugar did not acetylate well, did not run on the nonpolar DB5 column, but eluted finally after 14:05 (min:sec) from the ECNSS-M column, which is too late to be associated with peaks observed with AgC6 and AgH9 profiles. (Although not obvious from figures of elution profiles, all GLC runs were done for 20 min).

Mannans and glucans are modified by branched side chains usually carbohydrates (107). Based on evidence from GLC and mass spectroscopy data, the substance which modifies the mannan backbone of AgH9 may be inositol or a closely related compound.

As previously mentioned, mannan, in and of itself, reportedly suppresses immunity and is incapable of eliciting a humoral immune response (174). This would also indicate that an additional moiety is responsible for the antigenicity (and, perhaps, immunogenicity) of AgC6 and AgH9. Although I cannot rule out that these antigens were glycoproteins in situ, evidence speaks against it. Protease treatment and excess heat, both used in preparatory steps for antigen isolation, would

likely have destroyed proteins associated with the antigens. Although initial protein determinations indicated that the crude antigen extract contained approximately 29% protein, after HPLC no protein was detectable in the isolated antigen. If the antigens were originally glycoproteins, the protein portion is not required in the antigen-antibody reactions we have studied.

LITERATURE CITED

1. Al-Doory, Y. (1975). The Epidemiology of Human Mycotic Diseases. Charles C. Thomas, Publisher. Springfield, Illinois.
2. Albersheim, P., D.J. Nevins, P.D. English, and A. Karr. (1967). A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. Carbohydr. Res. 5: 340-345.
3. Ballman, G.E. and W.L. Chaffin. (1979). Lipid synthesis during reinitiation of growth from stationary phase cultures of Candida albicans. Mycopathologia 67: 39-43.
4. Ballou, C. (1976). Structure and biosynthesis of the mannan component of the yeast cell envelope. Advances Microbiol. Physiol. 14: 93-158.
5. Banerjee, U., L.N. Mohapatra, and R. Kumar. (1984). Role of antibody against murine candidiasis. Indian J. Med. Res. 79: 760-765.
6. Benbouzid, A., and R. Guinet. (1984). Purification of soluble antigens of systemic candidiasis by antibody affinity chromatography. J. Chromatography 306: 117-124.
7. Berntsson, E. (1984). Antibodies to Candida albicans in healthy, colonized, and infected persons. Mykosen 27: 443-451.
8. Bishop, C.T., F. Blank, and P.E. Gardner. (1960). The cell wall polysaccharides of Candida albicans: glucan, mannan, and chitin. Can. J. Chem. 38: 869-881.
9. Bodey, G.P. (1984). Candidiasis in cancer patients. Am. J. Med. 77: 13-19.
10. Braun, P.C., and R.A. Calderone. (1978). Chitin synthesis in Candida albicans: comparison of yeast and hyphal forms. J. Bacteriol. 135: 1472-1477.
11. Brawner, D.L., and J.E. Cutler. (1984). Variability in expression of a cell surface determinant on Candida albicans as evidenced by an agglutinating monoclonal antibody. Infect. Immun. 43: 966-972.

12. Brenkle, R. and R. Kornfeld. (1980). Structure of the oligosaccharide of mouse IgM secreted by the MOPC 104E plasmacytoma. Arch. Biochem. Biophys. 201: 160-173.
13. Brown, J.P. (1971). Susceptibility of the cell walls of some yeasts to lysis by enzymes of Helix pomatia. Can. J. Microbiol. 17: 205-208.
14. Brown, L.A. and W.L. Chaffin. (1981). Differential expression of cytoplasmic proteins during yeast bud and germ tube formation in Candida albicans. Can. J. Microbiol. 27: 580-585.
15. Brummel, M. and D.R. Soll. (1982). The temporal regulation of protein synthesis during synchronous bud or mycelium formation in the dimorphic yeast Candida albicans. Dev. Biol. 89: 211-284.
16. Buckley, R.E., B. Hattler, C. Emijewski, and D. Amos. (1968). Cellular immunity associated with chronic mucocutaneous moniliaiasis and recurrent staphylococcal botryomycosis: immunological reconstitution by allogeneic bone marrow. Clin. Exp. Immunol. 3: 159-169.
17. Burges, G., H.P. Holley, and G. Virella. (1983). Circulating immune complexes in patients with Candida albicans infections. Clin. Exp. Immunol. 53: 165-174.
18. Cabib, E., M.S. Kang, B. Bowers, J. Au-Young, M. Slater, P.W. Robbins. (1985). Chitin synthetase and formation of primary septum in fungal cell wall components: biosynthesis and localization. 85th Annual ASM Meeting, Las Vegas.
19. Casal, M. and M.J. Linares. (1983). Contribution to the study of the enzymatic profiles of yeast organisms with medical interest. Mycopathologia 81: 155-159.
20. Cassone A., N. Simonetti, and V. Strippoli. (1973). Ultrastructural changes in the wall during germ tube formation from blastospores of Candida albicans. J. Gen. Microbiol. 77: 417-426.

21. Cassone, A., E. Mattia, and L. Boldrini. (1978). Agglutination of blastospores of Candida albicans by concanavalin A and its relationship with the distribution of mannan polymers and the ultrastructure of the cell wall. J. Gen. Microbiol. 105: 263-273.
22. Cassone, A., E. Mattia, and L. Boldrini. (1978). Agglutination of blastospores of Candida albicans by concanavalin A and its relationship with the distribution of mannan polymers and the ultrastructure of the cell wall. J. Gen. Microbiol. 105: 263-273.
23. Chattaway, F.W., M.R. Holmes, and A.J.E. Barlow. (1968). Cell wall composition of the mycelial and blastospores of Candida albicans. J. Gen. Microbiol. 51: 367-376.
24. Chaffin, W.L. (1983). The relationship between yeast cell size and cell division in Candida albicans Can. J. Microbiol. 30: 192-303.
25. Chesebro, B., K. Wehrly, M. Cloyd, W. Britt, J. Portis, J. Collins and J. Nisho. (1981). Characterization of mouse monoclonal antibodies specific for Friend Leukemia Virus-induced erythroleukemia cells: Friend-specific antigens. Virology 112: 131-144.
26. Chiew, Y.Y., M.G. Shepherd, and P.A. Sullivan. (1980). Regulation of chitin synthesis during germ-tube formation in Candida albicans. Arch. Microbiol. 125: 97-104.
27. Cleveland, W., B. Fogel, W. Brown, and H. Kay. (1968). Foetal thymic transplant in a case of Digeorge syndrome. Lancet ii: 1211.
28. Cutler, J.E. and A.H. Poor. (1981). Effect of mouse phagocytes on Candida albicans in in vitro chambers. Infect. Immun. 31: 1110-1116.
29. Cutler, J.E. and R. Lloyd. (1982). Enhanced antibody responses induced by Candida albicans in mice. Infect. Immun. 38: 1102-1108.

30. Cutler, J.E. and K.C. Hazen. (1983). Yeast/Mold morphogenesis in *Mucor* and *Candida albicans*. pp. 280-306. In *Secondary Metabolism and Differentiation in Fungi*. Edited by J.W. Bennett and A. Ciegler. Marcel Dekker Press, NY.
31. Dabrowa, N., D.H. Howard, J.W. Landau, and Y. Schechter. (1970). Synthesis of nucleic acids and proteins in the dimorphic forms of *Candida albicans*. *Sabouraudia* 8: 163-169.
32. Diamond, R.D., and R. Krzesicki. (1978). Mechanisms of attachment of neutrophils to *Candida albicans* pseudohyphae in the absence of serum, and of subsequent damage to pseudohyphae by microbicidal processes of neutrophils *in vitro*. *J. Clin. Invest.* 61: 360-369.
33. Diamond, R.D., R. Kresicki, and W. Jao. (1978). Damage to pseudohyphal forms of *Candida albicans* by neutrophils in the absence of serum *in vitro*. *J. Clin. Invest.* 61: 340-359.
34. Djaczenco, W., and A. Cassone. (1971). Visualization of new ultrastructural components in the cell wall of *Candida albicans* with fixatives containing TAPO. *J. Cell Biol.* 52: 186-190.
35. Domer, J.E. and S. Moser. (1978). Experimental murine candidiasis: cell mediated immunity after cutaneous challenge. *Infect. Immun.* 20: 88-98.
36. Dubois, M., K.A. Giles, J.K. Hamilton, P.A. Rebers, and F.A. Smith. (1956). Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28: 350-356.
37. Durandy, A., A. Fisher, and C. Griscella. (1983). Specific *in vitro* antimannan-rich antigen of *Candida albicans* antibody produced by sensitized human blood lymphocytes. *J. Clin. Invest.* 71: 1602-1613.
38. Emmons, C.W., C.H. Binford, J.P. Utz, and K.J. Kwon-Chung. (1977). *Medical Mycology*, 3rd edition. Lea and Febiger. Philadelphia, PA.
39. Evans, E.G.V., F.C. Odds, M.D. Richardson, and K.T. Holland. (1975). Optimum conditions for initiation of filamentation in *Candida albicans*. *Can. J. Microbiol.* 21: 338-342.

40. Fisher, A., J. Ballet, and J. Griscelli. (1978). Specific inhibition of in vitro Candida-induced lymphocyte proliferation by polysaccharidic antigen present in serum of patients with chronic mucocutaneous candidiasis. J. Clin. Invest. 62: 1005-1013.
41. Fisher, A., L. Pichat, M. Audinot, and C. Griscelli. (1982). Defective handling of mannan by monocytes in patients with chronic mucocutaneous candidiasis resulting in specific cellular unresponsiveness. Clin. Exp. Immunol. 47: 653-660.
42. Fukazawa, Y., T. Shinoda, and T. Tsuchiya. (1968). Response and specificity of antibodies of Candida albicans. J. Bacteriol. 95: 754-763.
43. Garvey, J.S., N.E. Cremer, and D. H. Sussdorf. (1977). Gel Diffusion and Gel Electrophoresis, p. 218-219 and 313-335. in Methods in Immunology, 3rd edition., W.A. Benjamin, Inc., Reading, MA.
44. Good, R.A., J. Finstad, and R.A. Gatti. (1970). Bullwarks of body defense, pp. 76-114. in S. Mudd (ed.) Infectious Agents and Host Reactions. W.B. Saunders Co., Philadelphia.
45. Gold, J.W.M., B. Wong, E.M. Bernard, T.E. Kiehn, and D. Armstrong. (1983). Serum arabinitol concentrations and arabinitol/creatinin ratios in invasive candidiasis. J. Infect. Dis. 147: 504-513.
46. Gow, A.R., and G.W. Gooday. (1982). Vacuolation, branch production and linear growth of germ tubes of Candida albicans. J. Gen. Microbiol. 128: 2195-2198.
47. Gow, A.R. and G.W. Gooday. (1984). A model for the germ tube formation and mycelial growth form of Candida albicans. Sabouraudia 22: 137-143.
48. Greenfield, R.A. and J.M. Jones. (1982). Comparisons of cytoplasmic extracts of eight Candida species and Saccharomyces cerevisiae. Infect. Immun. 35: 1157-1161.
49. Guinet, R.M.F. and S.M. Gabriel. (1980). Candida albicans Group A specific soluble antigens demonstrated by quantitative immunoelectrophoresis. Infect. Immun. 29: 853-858.

50. Hasenclever, H.F. and W.O. Mitchell. (1961). Antigenic studies of *Candida*. I. Observation of two antigenic groups in *Candida albicans*. *J. Bacti.* 82: 570-573.
51. Hasenclever, H.F. and W.O. Mitchell. (1964). Immunochemical studies on polysaccharides of yeast. *J. Immunol.* 93: 763-771.
52. Hazen, K.C. and J.E. Cutler. (1979). Autoregulation of germ tube formation by *Candida albicans*. *Infect. Immun.* 24:661-666.
53. Hazen, K. C. and J.E. Cutler. (1983). Effect of cobalt and morphogenic autoregulatory substance (MARS) on morphogenesis of *Candida albicans*. *Exp. Mycology* 7: 182-187.
54. Hazen, K. C. and J.E. Cutler. (1983). Isolation and purification of morphogenic autoregulatory substance produced by *Candida albicans*. *J. Biochem.* 94: 777-783.
55. Hector, R.F., J.E. Domer, and E.W. Carrow. (1982). Immune responses to *Candida albicans* in genetically distinct mice. *Infect. Immun.* 38: 1020-1028.
56. Heden, D.M. and J.D. Buck. (1980). A reemphasis-germ tubes diagnostic for *Candida albicans* have no constrictions. *Mycopathologia* 70: 95-101.
57. Heller, S.R. and G.W.A. Milne. EPA/NIH Mass Spectral Data Base. US Department of Commerce, National Bureau of Standards. Washington D.C.
58. Herman, M.A., and D.R. Soll. (1984). A comparison of volume growth during bud and mycelium formation in *Candida albicans*: a single cell analysis. *J. Gen. Microbiol.* 130: 2219-2228.
59. Hether, N.W., P.A. Campbell, L.A. Baker, and L.L. Jackson. (1983). Chemical composition of abiological functions of *Listeria monocytogenes* cell wall preparations. *Infect. Immun.* 39: 1114-1121.
60. Hiatt, W.R., C.B. Inderleid, and P.S. Sypherd. (1980). Differential synthesis of polypeptides during morphogenesis of *Mucor*. *J. Bacti.* 141: 1350-1359.

61. Hoeprich, P.D. (1983). Infectious Diseases. 3rd edition, Harper and Row, Philadelphia.
62. Holliday, M.G. (1979). A modified cytoplasmic antigen of Candida albicans for serodiagnosis of systemic candidiasis. J. Immunol. Methods 31: 71-81.
63. Jones, G.H., and C.E. Ballou. (1969). Studies on the structure of yeast mannan. II. Mode of action of the Arthrobacter alpha-mannosidase on yeast mannan. J. Biol. Chem. 244: 1052-1059.
64. Kakar, S.N., R.M. Partridge, and P.T. Magee. (1983). A genetic analysis of Candida albicans: isolation of a wide variety of auxotrophs and demonstration of linkage and complementation. Genetics 104: 241-255.
65. Karwowska, W., G. de St. Basile, and B. Lisowska-Grospierre. (1984). Antibody levels to Candida albicans carbohydrate and major cytoplasmic antigens isolated from standard and patient strains. Ann. Immunol. 135D: 145-159.
66. Kessler, G. and W. J. Nickerson. (1959). Glucomannan-protein complexes form cell walls of yeasts. J. Biol. Chem. 234: 2281-2285.
67. Kirkpatrick, C.H., J.W. Chandler, and R.N. Schimke. (1970). Chronic mucocutaneous moniliasis with impaired delayed hypersensitivities. Clin. Exp. Immunol. 6: 375-385.
68. Kirkpatrick, C.H., R.R. Rich, and J.E. Bennett. (1971). Chronic mucocutaneous candidiasis: model-building in cellular immunity. Ann. Intern. Med. 74: 955-978.
69. Kirkpatrick, C.H., R.R. Rich, and T.K. Smith. (1972). Effects of transfer factor on lymphocyte function in anergic patients. J. Clin. Invest. 51: 2948-2958.
70. Kirkpatrick, C.H. and T.K. Smith. (1974). Chronic mucocutaneous candidiasis: immunologic and antibiotic therapy. Ann. Intern. Med. 80: 310-320.
71. Kohler, R., L.J. Wheat, and A. White. (1979). Detection of IgG Candida antibodies by solid-phase radioimmunoassay and comparison with agar gel diffusion. Am. J. Med. Sc. 278: 49-55.

72. Kocourek, J. and C.E. Ballou. (1969). Method for fingerprinting yeast cell wall mannans. J. Bacteriol. 100: 1175-1180.
73. Kwon-Chung, K.J. and W.B. Hill. (1970). Studies on the pink, adenine-deficient strains of Candida albicans. I. Cultural and morphological characteristics. Sabouraudia 8: 48-59.
74. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.
75. Land, G.A., W.C. McDonald, R.L. Stjernholm, and L. Friedman. (1975). Factors affecting filamentation in Candida albicans: relationship of uptake and distribution of proline to morphogenesis. Infect. Immun. 11: 1014-1023.
76. Lee, K.L., H.R. Buckley, and C.C. Campbell. (1975). An amino acid liquid synthetic medium for mycelial and yeast forms of Candida albicans. Sabouraudia 13: 148-153.
77. Lehrer, R.I. (1972). Functional aspects of a second mechanism of candidacidal activity by human neutrophils. J. Clin. Invest. 51: 2566-2572.
78. Lehrer, N., E. Segal, and L. Barr-Nea. (1983). In vitro and in vivo adherence of Candida albicans to mucosal surfaces. Ann. Microbiol. 134B: 293-306.
79. Lingappa, B.T., and Y. Lingappa. (1966). The nature of self inhibition of germination of conidia of Glomerella cingulata. J. Gen. Microbiol. 43: 91-100.
80. Lingappa, B.T., M. Prasad, Y. Lingappa, D.F. Hunt, and K. Biemann. (1969). Phenylethyl alcohol and tryptophol: autoantibodies produced by the fungus C. albicans. Science 163: 192-194.
81. Louria, D.B. and R.G. Brayton. (1964). Behavior of Candida cells within leukocytes. Proc. Soc. Exp. Biol. Med. 115: 93-98.
82. Lura, L.G. (ed). (1968). Manual of Histological Stains, Methods of the Armed Forces Institute of Pathology. 3rd edition. McGraw-Hill Inc., New York.

83. Mackler, M.T., A.J. Pesce, J.A. Campbell, and T.R. Green. (1981). Application of ELISA and ELADA (double substrate immunoassay) to red blood cell antigens. *Transfusion* 21: 303-312.
84. Mackler, M.T., C.R. Mlecko, and A.J. Pesce. (1981). Immunoenzymometric assays for simultaneous quantitation and distribution of erythrocyte antigen. *Clin. Chem.* 27: 1609-1613.
85. Maisch, A.P., and R.A. Calderone. (1981). Role of surface mannan in the adherence of Candida albicans to fibrin-platelet clots formed in vitro. *Infect. Immun.* 32: 92-97.
86. Maksymiuk, A.W., S. Thongprasert, R. Hopfer, M. Luno, V. Fainstein, and G. Bodey. (1984). Systemic candidiasis in cancer patients. *Am. J. Med.* 77: 20-27.
87. Manning, M. and T.G. Mitchell. (1980). Analysis of cytoplasmic antigens of the yeast and mycelial phases of Candida albicans by two dimensional gel electrophoresis. *Infect. Immun.* 30: 484-495.
88. Manning, M. and T.G. Mitchell. (1980). Strain variation and morphogenesis of yeast and mycelial phase Candida albicans in low-sulfate, synthetic medium. *J. Bacteriol.* 142: 714-719.
89. Manning, M. and T.G. Mitchell. (1980). Morphogenesis of Candida albicans and cytoplasmic proteins associated with differences in morphology, strain, or temperature. *J. Bacteriol.* 144: 258-273.
90. Mardon, D., E. Balish, and A.W. Phillips. (1969). Control of dimorphism in a biochemical variant of Candida albicans. *J. Bacteriol.* 100: 701-707.
91. Marriott, M.S. (1975). Isolation and chemical characterization of plasma membranes from the yeast and mycelial forms of Candida albicans. *J. Gen. Microbiol.* 86: 115-132.
92. Mattia, E., G. Carruba, L. Amgiollella, and A. Cassone. (1982). Induction of germ tube formation by N-actyl-D-glucosamine in Candida albicans: uptake of inducer and germinative response. *J. Bacteriol.* 152: 555-562.

93. McCourtie, J. and J. Douglas. (1981). Relationship between cell surface composition of Candida albicans and adherence to acrylic after growth on different carbon sources. Infect. Immun. 32: 1234-1241.
94. McGinnis, M.R. (1980). Laboratory Handbook of Medical Mycology. Academic Press, Inc. New York.
95. Meinhof, W. (1982). Demonstration of typical features of individual Candida albicans strains as a means of studying infection. Chemotherapy 28: 51-55.
96. Merz, W.G., G.L. Evans, S. Shadomy, S. Anderson, L. Kaufman, P. Kozinn, D.W. MacKenzie, W. Protzman, and J.S. Remington. (1977). Laboratory evaluation of serological tests for systemic candidiasis: a cooperative study. J. Clin. Microbiol. 5: 596-603.
97. Mitchell, T.G. (1980). Opportunistic Mycoses. in Zinsser Microbiology. Eighteenth edition. W.K. Joklik, H.P. Willett, and D.B. Amos (eds). Appleton-Century-Crofts, New York.
98. Montrocher, R. (1980). Significance of immunoprecipitation in yeast taxonomy: antigenic analyses of some species within the genus Candida. Cell Mol. Biol. 26: 293-302.
99. Mooney, D.T. and P.S. Sypherd. (1976). Volatile factor involved in dimorphism of Mucor racemosus. J. Bacteriol. 126: 1266-1270.
100. Morelli, H. and L.T. Rosenberg. (1971). The role of complement during experimental Candida infections in mice. Infect. Immun. 27: 376-386.
101. Moser, S.A. and J. E. Domer. (1980). Effects of cyclophosphamide on murine candidiasis. Infect. Immun. 27: 376-386.
102. Mourad, S., and L. Friedman. (1961). Pathogenicity of Candida. J. Bacteriol. 81: 550-556.
103. Mourad, S., and L. Friedman. (1968). Passive immunization of mice against Candida albicans. Sabouraudia 6: 103-105.
104. Muller, H.L. and G. Kirchhof. (1969). Serologische Typen von Candida albicans. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. 1: Orig. Reihe A. 210: 114-121.

105. Nickerson, W.J. (1953). Reduction of inorganic substances by yeast. I. Extracellular reduction of sulfate by species of Candida. J. Infect. Dis, 93: 43-56.
106. Odds, F.C., E.G.V. Evans, M.A.R. Taylor, and J.K. Wales. (1978). Prevalence of pathogenic yeasts and humoral antibodies to Candida in diabetic patients. J. Clin. Path. 31: 840-844.
107. Odds, F.C. (1979). Candida and Candidosis. University Park Press, Baltimore.
108. Odds, F.C., and A.B. Abbott. (1980). A simple system for the presumptive identification of Candida albicans and the differentiation of strains within the species. Sabouraudia 18: 301-317.
109. Ofek, I, and E.H. Beachey. (1980). General concepts and principles of bacterial adherence in animals and man. in Bacterial Adherence, edited by E.H. Beachey, Chapman and Hall, London. pp. 1-29.
110. Olaiya, A.G., and S.J. Sogin. (1979). Ploidy of Candida albicans. J. Bacteriol. 140: 1043-1049.
111. Olaiya, A.G., J.R. Steed, and S.J. Sogin. (1980). Deoxyribonucleic acid-deficient strains of Candida albicans. J. Bacteriol. 141: 1284-1288.
112. Orlowski, M., and P.S. Sypherd. (1976). Cyclic guanosine 3'5'-monophosphate in the dimorphic fungus Mucor racemosus. J. Bacteriol. 125: 1226-1228.
113. Pabst, H.F. and R. Swanson. (1972). Successful treatment of candidiasis with transfer factor. Br. Med J. 2: 442-443.
114. Paterson, P., R. Semo, B. Blumenschein, and J. Swelstad. (1971). Mucocutaneous candidiasis anergy: plasma cell inhibitor of cellular immunity after amphotericin B therapy. Clin. Exp. Immunol. 9: 595-602.
115. Pearsall, N., B. Adams, and R. Bunni. (1978). Immunologic responses to Candida albicans. III. Effects of passive transfer of lymphoid cells or serum on murine candidiasis. J. Immunol. 120: 1176-1180.

116. Pereira, H.A., and C.S. Hosking. (1984). The role of complement and antibody in opsonization and intracellular killing of Candida albicans. Clin. Exp. Immunol. 57: 307-314.
117. Peterson, E.M. and R.A. Calderone. (1977). Growth inhibition of Candida albicans by rabbit alveolar macrophages. Infect. Immun. 15: 910-915.
118. Phaff, H.J. (1971). Structure and biosynthesis of the yeast cell envelope. in The yeasts 2: 135-210. A.H. Rose and J.S. Harrison, eds. Academic Press, London.
119. Piccolella, E., G. Lombardi, and R. Morelli. (1980). Human lymphocyte activating properties of a purified polysaccharide from Candida albicans: B and T cell cooperation in the mitogenic response. J. Immunol. 125: 2082-2087.
120. Piccolella, E., G. Lombardi, and R. Morelli. (1981). Generation of suppressor cells in the response of human lymphocytes to a polysaccharide antigen from Candida albicans. J. Immunol. 126: 2151-2155.
121. Piccolella, E., G. Lombardi, and R. Morelli. (1981). Mitogenic response to a purified Candida albicans polysaccharide fraction: lack of helper activities is responsible for the in vitro unresponsiveness to a second antigenic challenge. J. Immunol. 126: 2156-2160.
122. Polonelli, L., C. Archibusacci, M. Sestito, and G. Morace. (1983). Killer system: a simple method for differentiating Candida albicans strains. J. Clin. Microbiol. 17: 774-780.
123. Poulain D., G. Tronchin, J.F. Dubremetz, and J. Biguet. (1978). Ultrastructure of the cell wall of Candida albicans blastospores: study of its constitutive layers by the use of a cytochemical technique revealing polysaccharides. Ann. Microbiol. 129: 141-153.
124. Poulain, D., G. Tronchin, B. Lefebvre, and M.O. Husson. (1982). Antigenic variability between Candida albicans blastospores isolated from healthy subjects and patients with Candida infection. Sabouraudia 20: 173-177.

125. Poulain, D., G. Tronchin, A. Vernes, R. Popeye, and J. Biguet. (1983). Antigenic variations of Candida albicans in vivo and in vitro: relationships between P antigens and serotypes. Sabouraudia 21: 99-112.
126. Poulter, R., K. Jeffery, M.J. Hubbard, M.G. Shepherd, and P.A. Sullivan. (1981). Parasexual genetic analysis of Candida albicans by spheroplast fusion. J. Bacteriol. 146: 833-840.
127. Poulter, R., V. Hanrahan, K. Jeffery, D. Markie, M.G. Shepherd, and P.A. Sullivan. (1982). Recombination analysis of naturally diploid Candida albicans. J. Bacteriol. 152: 969-975.
128. Pringle, J., A. Adams, and C.W. Jacobs. (1985). Mechanisms for localization of secretions and cell wall deposition in yeasts. in "Fungal cell wall components: biosynthesis and localization." 85th Annual ASM Meeting, Las Vegas.
129. Rebora, A., R.R. Marples, and A.M. Kligman. (1973). Experimental infection with Candida albicans. Arch. Dermatol. 108: 69-73.
130. Reiss, E., L. Stockman, R.J. Kuykendall, and S.J. Smith. (1981). Dissociation of mannan-antibody complexes and detection of Candida albicans mannan by enzyme immunoassay variations. Clin. Chem. Microbiol. 28: 306-310.
131. de Repentigny, L., R. Kuykendall, F. Chandler, J.R. Broderson, and E. Reiss. (1984). Comparison of serum mannan, arabinitol, and mannose in experimental candidiasis. J. Clin. Microbiol. 19: 804-812.
132. Riggsby, W.S., L.J. Torres-Bauza, J.W. Wills, and T.M. Townes. (1982). DNA content, kinetic complexity, and the ploidy question in Candida albicans. Mol. Cell. Biol. 2: 853-862.
133. Rinaldi, M. (1982). Use of potato flakes agar in clinical mycology. J. Clin. Microbiol. 15: 1159-1160.
134. Rippon, J.W. (1980). Dimorphism in pathogenic fungi. CRC Critical Rev. Microbiol. 8: 49-97.

135. Samaranayake, L., and T.W. MacFarlane. (1982). The effect of dietary carbohydrates on the in vitro adhesion of Candida albicans to epithelial cells. J. Med. Microbiol. 15: 511-517.
136. Sarachek, A., D.D. Rhoads, and R.H. Schwartzhoff. (1981). Hybridization of Candida albicans through fusion of protoplasts. Arch. Microbiol. 129: 1-8.
137. Scherwitz, C., R. Martin, and H. Ueberberg. (1978). Ultrastructural investigations of the formation of Candida albicans germ tubes and septa. Sabouraudia 16: 115-124.
138. Schreiber, J.R., E. Maynard, and M. Lew. (1984). Candida antigen detection in two premature neonates with disseminated candidiasis. Pediatrics 74: 838-841.
139. Schulkind, M.L., W.L. Adler, W.A. Altemeier, and E.M. Ayoub. (1972). Transfer factor in the treatment of a case of chronic mucocutaneous candidiasis. Cell Immunol. 3: 606-615.
140. Segal, E., N. Vardinon, J. Schwartz, and E. Eylan. (1975). Antigenic relationships between Candida albicans and various yeasts as detected by immunoglobulin-class specificity. Path. et Microbiol. 42: 494-508.
141. Segal, E., A. Soroka, and A. Schechter. (1984). Correlative relationship between adherence of Candida albicans to human vaginal epithelial cells in vitro and candidal vaginitis. Sabouraudia 22: 191-200.
142. Shepherd, M.G., Y.Y. Chiew, S.P. Ram, and P.A. Sullivan. (1980). Germ tube induction in Candida albicans. Can. J. Microbiol. 26: 21-26.
143. Simonetti, N., V. Strippoli, and A. Cassone. (1974). Yeast mycelial conversion induced by N-acetyl-d-glucosamine in Candida albicans. Nature 250: 344-346.
144. Skeryl, K.G., R.A. Calderone, E. Segal, T. Sreevalsan, and W. M. Scheld. (1984). In vitro binding of Candida albicans yeast cells to human fibronectin. Can. J. Microbiol. 30: 221-227.

145. Smail, E.W. and J.M. Jones. (1984). Demonstration and solubilization of antigens expressed primarily on the surfaces of Candida albicans germ tubes. Infect. Immun. 45: 74-81.
146. Soll, D.R. and G.W. Bedell. (1978). Bud formation and the inducibility of pseudomycelium outgrowth during release from stationary phase in Candida albicans. J. Gen. Microbiol. 108: 173-180.
147. Spurr, A.R. (1969). A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastructural Res. 26: 31-38.
148. Stewart, T.S. and C.E. Ballou. (1968). A comparison of yeast mannans and phosphomannans by acetolysis. Biochemistry 7: 1855-1863.
149. Stewart-Tull, D.E.S., W.K. Timperley, and C.H. Horn. (1966). Some immunochemical observations of the cell walls of yeasts. Sabouraudia 5: 104-109.
150. Sullivan, P.A., N.J. McHugh, L. Romana, and M.G. Shepherd. (1984) The secretion of N-acetylglucosaminidase durin germ-tube formation in Candida albicans. J. Gen. Microbiol. 130: 2213-2218.
151. Summers, D.F., A.P. Grollman, and H.F. Hasenclever. (1963). Polysaccharide antigens of candida cell wall. J. Immunol. 92: 491-499.
152. Sundarum, S., P.A. Sullivan, and M.G. Shepherd. (1981). Changes in lipid composition during starvation and germ tube formation in Candida albicans. Exp. Cell Res. 116: 207-215.
153. Sundstrom, P.M. and G.E. Kenny. (1984). Characterization of antigens specific to the surface of germ tubes of Candida albicans by immunofluorescence. Infect. Immun. 43: 850-855.
154. Suzuki, K., Y. Okawa, K. Hashimoto, S. Suzuki, and M. Suzuki. (1984). Protecting effect of chitin and chitosan on experimentally induced murine candidiasis. Microbiol. Immunol. 28: 903-912.
155. Suzuki, M. and Y. Fukazawa. (1982). Immunochemical characterization of Candida albicans cell wall antigens: specific determinant of Candida albicans serotype A mannan. Microbiol. Immunol. 26: 387-402.

156. Sweet, C.E. and L. Kaufman. (1970). Application of agglutinins for the rapid and accurate identification of medically important Candida sp. Appl. Microbiol. 19: 830-835.
157. Taguchi, M., M. Tsukiji, and T. Tsuchiya. (1979). Rapid identification of yeasts by serological methods; a combined serological and biological method. Sabouraudia 17: 185-191.
158. Takeda, N., Y. Okubo, T. Ichikawa, and S. Suzuki. (1979). Cross reaction between mycelial galactomannan of three Hormodendrum strains and the mannans of two Candida albicans strains of different serotypes A and B. Infect. Immun. 23: 146-149.
159. Terenzi, H.F., and R. Storck. (1969). Stimulation of fermentation and yeast-like morphogenesis in Mucor rouxii by phenylethyl alcohol. J. Bacteriol. 97: 397-403.
160. Trinci, A.P.J. (1971). Exponential growth of the germ tubes of fungal spores. J. Gen. Microbiol. 67: 345-348.
161. Tronchin, G., D. Poulain, and A. Vernes. (1984). Cytochemical and ultrastructural studies of Candida albicans. III. Evidence for modifications of the cell wall coat during adherence to human buccal epithelial cells. Arch. Microbiol. 139: 221-224.
162. Tsuchiya, T., Y. Fukazawa, and S. Kawakita. (1959). A method for the rapid identification of the genus Candida. Mycopathologia 10: 191-206.
163. Tsuchiya, T., Y. Fukazawa, M. Taguchi, T. Nakase, and T. Shinoda. (1974). Serological aspects of yeast classification. Mycopathologia 53: 77-91.
164. Valdimarsson, H.J., R. Higgs, R. Wells, M. Yamamura, J. Hobbs, and P. Holt. (1973). Immune abnormalities associated with chronic mucocutaneous candidiasis. Cell. Immunol. 6: 384-361.
165. Vaughn, V.J. and E.D. Weinberg. (1968). Candida albicans dimorphism and virulence: Role of copper. Mycopathologia 58: 27-29.

166. Wain, W.H., M.F. Price, A.R. Brayton, and R.A. Cawson. (1976). A re-evaluation of the effect of cysteine on Candida albicans. Sabouraudia 13: 74-82.
167. Warburg, O. and W. Christian. (1942). Isolierung und Kristallization des Garlungs ferments Enolase. Biochem. Z. 310: 384-421.
168. Warnock, D.W., D.C. Speller, J.K. Day, and A.J. Farrell. (1979). Resistogram method for differentiation of strains of Candida albicans. J. Appl. Bacteriol. 46: 571-578.
169. Watkins, C.D., P.M. Brandt, and D.O. McClary. (1974). Selection and characterization of acriflavine-induced mutants of Candida albicans. Antonie von Leeuwenhoek J. Microbiol. Serol. 40: 153-160.
170. Whelan, W.L., R.M. Parttridge, and P.T. Magee. (1980). Heterozygosity and segregation in Candida albicans. Mol. Gen. Genet. 180: 107-113.
171. Whelan, W.L., and P.T. Magee. (1981). Natural heterozygosity in Candida albicans. J. Bacteriol. 145: 896-903.
172. Wickerham, L.J., and L.F. Rettger. (1939). A taxonomic study of Monilia albicans with special emphasis on morphology and morphological variations. J. Trop. Med. Hyg. 42: 204-216.
173. Winterrowd, G.E. and J.E. Cutler. (1983). Candida albicans-induced agglutinins and immunoglobulin E responses in mice. Infect. Immun. 41: 33-38.
174. Wirz, M. G. Lombardi, O. Pugliese, R. Morelli, and E. Piccolella. (1984). A purified polysaccharide isolated from Candida albicans induces antibody responses in vitro by human peripheral blood lymphocytes and discriminates between sera from normal and Candida albicans-infected individuals. Clin. Immunol. Immunopath. 33: 199-209.
175. Wolter, J.R. (1962). Endogenous fungus endophthalmitis. Arch. Ophthalmol. 68: 73-76.
176. Yamaguchi, H. (1974). Mycelial development and chemical alteration on Candida albicans from biotin insufficiency. Sbaouraudia 112: 320-328.

177. Yamaguchi, H. (1975). Control of morphism in Candida albicans by zinc: Effect on cell morphology and composition. J. Gen. Appl. Microbiol. 86: 370-372.
178. Yu, R.J., C.T. Bishop, F.P. Cooper, H.F. Hasenclever, and F. Blank. (1967). Structural studies of mannans from Candida albicans (serotypes A and B), Candida parapsilosis, Candida stellatoidea, and Candida tropicalis. Can. J. Chem. 45: 2205-2211.
179. Yu, R.J. C.T. Bishop, F.P. Cooper, F. Bland, and H.F. Hasenclever. (1967). Glucans from Candida albicans (serotype B) and from Candida parapsilosis. Can. J. Chem. 45: 2264-2267.

MONTANA STATE UNIVERSITY LIBRARIES



3 1762 10005065 5

D378
B739
cop.2