GERM TUBE ADHESINS

OF *CANDIDA ALBICANS*

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Dedicated to my Mother and to the memory of my Father

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SUMMARY

The object of the research described in this thesis was to characterize the germ tube adhesins of *Candida albicans*. Except for a few experiments with a germ tube-deficient mutant and its corresponding wild-type strain, all the experiments were done with *C. albicans* strain GDH 2346, already extensively used for adhesion studies in this laboratory.

From the numerous culture media for germ tube production, described in the literature, three were chosen for comparative study. Medium 199 and Glucose-Glycine Medium at 37°C were highly satisfactory and gave *C. albicans* 90 % conversion of yeast cells to hyphal-form cells. Sucrose-Gelatine medium gave only *C. albicans* 15% conversion. Temperature and pH also were influential variables: medium 199 at pH 6.7 and 37°C gave germ tubes, while the same medium at 22°C yielded only budding yeast cells. The same medium at pH 4.0 and 37°C likewise gave only yeast cells.

The first experiments on adhesins focused on the production and detection of mannoprotein adhesin (MPA) of the hyphal-form cells on plastic petri dishes. This allowed the isolation of MPA and the demonstration that it did not inhibit the adhesion of yeast-form *C. albicans* to buccal epithelial cells. This observation had not previously been reported in the literature. Using Concanavalin A-coated latex microspheres, MPA was detected on the plastic surface on which the *C. albicans* produced germ tubes. The adhesins were extracted with dithiothreitol and iodoacetamide treatment. This suggested that MPA of the hyphal-form cells on the plastic was not identical to that on yeast cells which in turn was involved in attachment to epithelial cells.

The main part of the research was directed at investigation of the lectin-like adhesins of hyphal-form *C. albicans* with fluorescent probes. These were neoglycoproteins consisting of sugars (fucose, mannose, glucose, galactose, lactose) convalently linked to BSA, which itself was labelled with fluorescein. Qualitative observations were made by conventional fluorescence microscopy and quantitative observations by fluorescence microscopy with image analysis, and also by

spectrofluorimetry and flow cytometry. This is belived to be first study in which all of these methods have been applied to *C. albicans* .

In conventional fluorescence microscopy, the hyphal-form cells bound both the fucose and mannose probes, but the latter only weakly. The fucose probe bound to both the yeast and germ tube portions of hyphal-form cells. Probes with glucose, galactose or lactose did not label the hyphal-form cells.

Semi-quantitation of the lectin-like adhesins by fluorescence microscopy with image analysis confirmed the efficient binding of the fucose probe. Germ tube portions of the hyphal-form cells, especially the central region, had higher fluorescence than yeast-cell portions. As before, the mannose probe bound less effectively than the fucose probe, while the glucose, galactose and lactose probes showed little or no binding.

Application of spectrofluorimetry to the study of fucose-probe binding allowed the quantitation of lectin-like adhesins during germ tube production to be monitored. Fucose-probe binding to the cells was also shown to be optimum at pH 5.0 in citrate buffer, to be time dependent and to be approaching saturation at 100 $\mu g\ ml^{-1}$. There appeared to be no requirement for Ca^{++} and Mg^{++} in fucose-probe binding. Attempts to inhibit fucose-probe binding by Fuc-BSA (without the fluorescein label) gave inconsistent results. As before, the mannose probe bound much less well than the fucose probe, but apparently better than the probes with the other sugars. One of the complications of the spectrofluorimetric studies was the need for pronase treatment to solubilize fluorescence and to allow the cells to be separated by centrifugation.

The final observations on fucose-binding were made by flow cytometry which for the main part confirmed the results obtained by the other methods. An exception was the effect of pH which by flow cytometry was as strong at pH 7.2 as at pH 5.0. Additional results obtained only by flow cytometry were: Glucose-Glycine Medium yielded hyphal-form cells that gave stronger fluorescence than cells in 199 Medium; 37°C was better than 22°C or 4°C for binding of the fucose probe to the hyphal-form

cells; and a germ tube-deficient mutant, when exposed to hyphal-form growth conditions for 2 h, showed much less binding of the fucose probe than the wild type which gave germ tubes. Confirmation of specificity and the need for a carrier molecule was obtained by showing that Fuc-BSA (without fluorescein) effectively inhibited the binding of the fucose probe, although L-fucose itself was inactive. BSA-FITC without fucose bound to both yeast and hyphal-form *C. albicans* .

In the final stages of the research, the binding of gold-labelled fibronectin to the yeast and hyphal-form cells was observed by transmission electron microscopy. The germ tube portion of the hyphal-form cells carried essentially all of the label, and attached yeast-cells portions and yeast-form cells remained unlabelled.

Future work in this area should focus on the molecular biology of adhesin molecules on the yeast and hyphal-form cells. The isolation and characterization of lectins may provide information on the possibility of preventing infection by blocking adhesion in humans. Neoglycoproteins could possibly be used as inhibitors to prevent colonization before microorganisms have had the chance to overwhelm the host.

DECLARATION

This thesis is the orginal work of the author except where otherwise stated

Gülhan Vardar Ünlü

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LIST OF ABBREVIATIONS

A	Absorbance
Au_{20}	Gold particle (diameter 20nm)
Au_{20}-Fn	Fibronectin coated gold particle
BBS	Borate buffer solution
BEC	Buccal epithelial cell
BSA	Bovine serum albumin
BSA-FITC	BSA labelled with FITC
°C	Degrees Celsius
C3d	Complement conversion product
Con A	Concanavalin A
Conc.	concentration
CR3	C3 receptor
DTT	Dithiothreitol
DW	Distilled water
EDC	1-Ethyl 3, 3-dimethylaminopropylcarbodiimide HCl
EP	Extracellular polymeric material
EDTA	Ethylenediamine tetra-acetic acid
EM	Electron microscopy
FCAU	Flow-cytometry arbitrary unit
FITC	Fluorecein isothiocyanate
FSC	Forward scatter light
Fn	Fibronectin
Fuc-BSA	Fucose coupled to BSA
Fuc-BSA-FITC	Fucose coupled to BSA and labelled with FITC
GG	Glucose-Glycine Medium
Glu-BSA-FITC	Glucose coupled to BSA and labelled with FITC
Gal-BSA	Galactose coupled to BSA

Gal-BSA-FITC	Galactose coupled to BSA and labelled with FITC
GT	Germ tube portion of hyphal form *C. albicans*
IAAU	Image analysis arbitrary unit
h	Hour
iC3b	Complement conversion product
kDa	Kilodaltons
l	Litre
Lac-BSA-FITC	Lactose coupled to BSA and labelled with FITC
Le^a	Lewis^a antigen
Le^b	Lewis^b antigen
LLA	Lectin-like adhesin
Man-BSA-FITC	Mannose coupled to BSA and labelled with FITC
MPA	Mannoprotein adhesin
min	Minute
ml	Millilitre
µg	Microgram
MLMwAA	Modified Lee Medium without amino acids
M	Molar
MWM	Molecular weight markers
NCYC	National Collection of Yeast Cultures
nm	Nanometer
NS	Not significant
P	Probability value
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
pH	Hydrogen ion concentration
rpm	Revolutions per minute
RGD	Arginine-glycine-aspartic acid peptide

SAP	secreted aspartyl proteinase
SDA	Sabouraud dextrose agar
SDS	Sodium dodecyl sulphate
sec	Second
SEM	Standard error of the mean
SFAU	Spectrofluorimetry arbitrary unit
SG	Sucrose-Gelatin Medium
SSC	Side scatter light
TEMED	N, N, N, N-tetramethyl-ethylenediamine
Tris	Tris (hydroxymetyl) aminomethane
Tris-HCl	Tris hydrochloride
TV	Television
VEC	Vaginal epithelial cells
v/v	Volume per volume ratio
YC	Yeast cell portion of hyphal form *C. albicans*
YNBGal	Yeast Nitrogen Base Medium containing galactose
w/v	Weight per volume ratio

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1. INTRODUCTION

1.1. HISTORY OF *CANDIDA ALBICANS*

The literature on disease caused by the organism now recognised as *Candida albicans* extends at least back to the 4th Century BC (Odds, 1988). Thus Hippocrates in his *Epidemics* described two cases of "oral aphthane", probably meaning thrush. The discovery of the actual microorganism that causes thrush was described wrongly by Langenberck (1839), in that he thought that the disease was caused by typhus. Berg (1846) was really the earliest author to describe in a detailed manner the relationship between the thrush fungus and mouth lesions. Wilkinson (1849) is credited with the first description of vaginal candidosis. The name *Oidium albicans* was suggested by Robin (1853) and was the first use of "*albicans*". In 1877, Grawitz (who, incidentally named the fungus *Mycoderma vini*) described both the budding yeast and the mycelial forms. Independently in 1887, Audrey proved definitively that the different morphological forms of the fungus were one and the same organism and that the morphology observed depended on the growth environment. This result was also confirmed by Roux and Linossier (1890).

The thrush fungus was the subject of confused taxonomic studies and for many years following its discovery it was linked with *Monilia* genus by Plaut (1887). Later he considered his isolate to be identical with the *Monilia albicans,* a thrush fungus as named by Zopf (1890). Afterwards and for several decades moniliasis was used as a name to describe infections due to the thrush fungus .

Castellani (1912) who first suggested that yeast species other than *M. albicans* might be involved in the pathogenesis of candidosis placed his several new species in the genus *Monilia.* Some of his species currently known as *C. guilliermondii, C. kefyr, C. krusei* and *C. tropicalis* were originially described under *Monilia.* This designation and the associated disease, moniliasis, was used until Berkhout (1923) proposed *Candida* as the generic name. As rotting fungi had been placed in *Monilia,* this latter genus was invalid for thrush fungus.

The thrush diseases, common in the 19th and 20th centuries, were associated with malnutrition (Berkhout, 1923). She also proposed the generic name "*Candida* " which derives from the white robe worn by *candidates* for the Senate, while *albicans* is the present participle of the Latin "*albicare* "(to whiten). Thus the combination *Candida albicans* essentially means "whitening white". The name *Candida* was adopted at the Third International Congress for Microbiology in 1939. In the above short historical section the author has relied extensively on Odds (1988) as a definitive source of information.

Since 1950, reports of superficial and systemic candidiasis have increased, and correlated with modern advances in chemotherapy and surgery. The increase has been associated with the use of broad-spectrum antibiotics, steroids and catheters (Ahearn, 1978). During the past decades, the organism has assumed increasing prominence as a major pathogen (Wade, 1993). Candidosis has been associated with cancer (Anaissie, 1992). The organism has become a serious pathogen of persons infected with the human immunodeficiency virus (HIV) who develop acquired immunodeficiency syndrome (AIDS). Full accounts are provided by Dupont *et al.* (1992) and Coleman *et al.* (1993).

1.2. BIOLOGY AND PATHOGENICITY OF *CANDIDA* SPECIES

1.2.1. Occurrence

Candida spp. are distributed ubiquitously in terrestrial and aquatic habitats and have been reported from domestic and wild animals. *C. albicans* has been more frequently recovered from animal hosts and especially human objects than any other *Candida* species (Odds, 1988).

C. albicans is a common commensal of the mouth and the gastrointestinal and vaginal tracts. However, a wide range of predisposing factors can lead to it producing candidosis in humans with superficial, deep-seated and disseminated

forms. Although *C. albicans* has been isolated from samples of soil, plants, the atmosphere and water, it is believed that these isolations may have come from human or animal contamination (Odds, 1988). *C. albicans* is frequently found in bedding, and washbasins but much less often on floors, window sills and other surfaces (Kashbur *et al.*, 1980). The organism survives poorly on dry surfaces such as skin or glass (Kashbur *et al.*, 1980), but moistened surfaces are suitable for recovering the yeast. Toothbrushes in long use by carriers of oral *Candida*, gave higher recoveries than from noncarriers (Koch and Koch, 1981). The yeast is also found in many types of hospital food samples, such as juices and soups where it also has been shown to grow (Wade, 1993) .

1.2.2. Classification

According to Rinaldi (1993), the systematic position of *C. albicans* is within the following taxonomic units: Kingdom *Fungi*, Division *Fungi Imperfecti*, Class *Blastomycetes*, Order *Crypococcales*, Family *Cryptococcaceae*, Genus *Candida*. The genus contains nearly 200 species which are separated on the basis of physiologic properties (Meyer *et al.*, 1984). A sexual state has not been reported in *C. albicans*. which is called an imperfect yeast, (Ahearn, 1978). Each species of the genus has a characteristic pattern of abilities and inabilities to assimilate various organic compounds and to ferment particular carbohydrates (Appendix 1).

Serological tests have also been used for determining the taxonomy of pathogenic yeasts. Hansenclever and Mitchell (1961) were the first to demonstrate that *C. albicans* isolates can be divided into serotypes A and B.

C. albicans is the most pathogenic species and there are two rapid and simple tests available for is identification: germ tube production in serum and chlamydospore formation in corn meal agar (Taschdjian *et al.*, 1960; Benham, 1931).

Antigenic cross-reactivity among pathogenic *Candida* species was shown by Tsuchiya *et al.* (1965). All *Candida* species possessed one common antigen and most possessed two. It was called 'Tsuchiya-factor" antisera and is available as a

commercial kit. The A serotype possesses the same group of antigens as serotype B plus the extra antigens, factor 6 and occasionally factor 13b (Tsuchiya *et al.*, 1965). Molecular techniques for distinguishing *C. albicans* strains have been described by Wilkinson *et al.* (1992).

1.2.3. Growth and Nutrition

The pathogenic yeasts are generally grown in media of pH 2.5 -7.5 and temperatures between 20 and 38°C (Odds, 1988). *Candida* species grow aerobically but are able to grow under condition of elevated CO_2 in air. However, an atmosphere of pure CO_2 inhibits their growth (Webster and Odds, 1987). All species of *Candida* assimilate and ferment glucose and none assimilates nitrate as a nitrogen source (Appendix 1). However, their abilities to use various additional carbon and nitrogen sources vary considerably (Meyer *et al.*, 1984). Most strains requires biotin and other vitamins for growth (Odds, 1988). This last reference provides a very full account of *Candida* growth and nutrition.

1.2.4. Cell Wall

The recognition of the *Candida* cell wall as a rigid structure which confers mechanical stability on the cell and maintains its characteristic shape can be traced back to Poulain *et al.* (1985) and Shepherd (1987). These and other authors (Sobel *et al.*, 1981; Samaranayake and MacFarlane, 1980) realized that it plays important biological roles in the adherence to host cells and inert surfaces. The cell wall has attracted interest as a potential target for novel antifungal agents, because of its glucan and chitin which are found in the yeast but not in the host (Bozzola *et al.*, 1984; Hilenski *et al.*, 1986).

The cell wall makes up approximately 30% of the dry weight of the cell and is a complex structure, approximately 100 to 400 nm thick in which distinct layers have been identified by differences in electron density (Cassone *et al.*, 1973, 1979). The number of layers and their morphology are variable and may be related to such factors

as: the stage of growth, the growth form (yeast or hyphal cells), the strain selected for study, the medium used to grow the cells, and the fixation procedure (Calderone and Braun, 1991). Most investigators have described five layers within the cell wall (Cassone *et al.*, 1973; Howlett and Squier, 1980). However, Poulain *et al.* (1985) have observed as many as eight or nine cell wall layers in *C. albicans*. These results covered a variety of wall types from cells grown on different media for various amounts of time. Cassone *et al.* (1979) showed that the cell wall layers of mannan gradually disappear after prolonged starvation of *C. albicans*.

The cell wall of *C. albicans* is composed primarily of the polysaccharides mannan, glucan and chitin (Figure 1). Mannan represent about 15 to 23 % of the yeast cell wall (dry weight) or about 40 % of the total cell wall polysaccharide. Glucans account for 47 to 60 % by weight of the cell wall. Proteins have been reported to comprise 6 to 25 %, lipids for 1 to 7 % and chitin for 0.6 to 9 % by weight of the cell wall (Calderone and Braun, 1991). The inner cell wall layers are composed of chitin and glucan (Shepherd, 1987). These components provide rigidity and appear essential for cell division. (Notario, 1982; Shepherd *et al.*, 1985). The distribution of these layers is similar in yeast cells, germ tubes and hyphae. However, during morphogenesis, the chitin and and certain kinds of glucans are quite different for yeast cells and germ tubes (Chattway *et al.*, 1968; Chiew *et al.*, 1980; Shepherd, 1987).

1.2.4.1. Mannoproteins

Chemical analysis has shown that the mannoprotein from *C. albicans* is similar to that of *Saccharomyces cerevisiae* and is schematically presented in Figure 2 (Shepherd and Gopal, 1991). Mannoproteins are not only found within the cell wall but also are located at the outer surface of the organisms and may be released into the culture medium. Two biotypes of *C. albicans* can be distinguished as serotypes A and B by serology based on differences in mannoprotein structures (Hasenclever and Mitchell, 1961). Mannan is characterized by three domains: an outer chain containing

Figure 1. Wall architecture of *C. albicans* (based on Calderone, 1993)

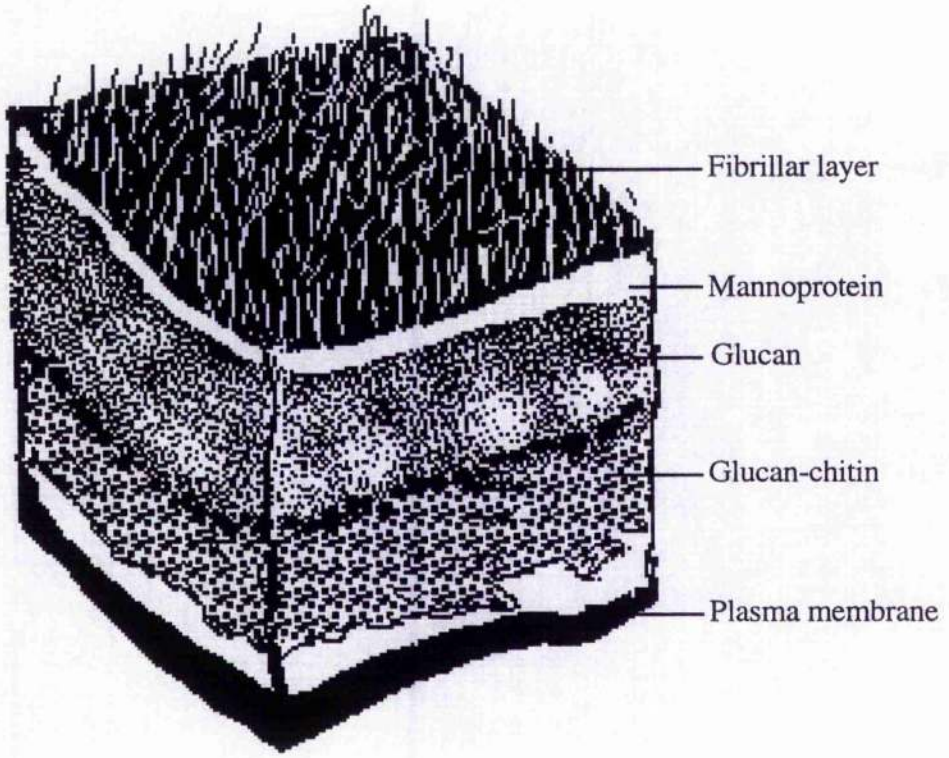
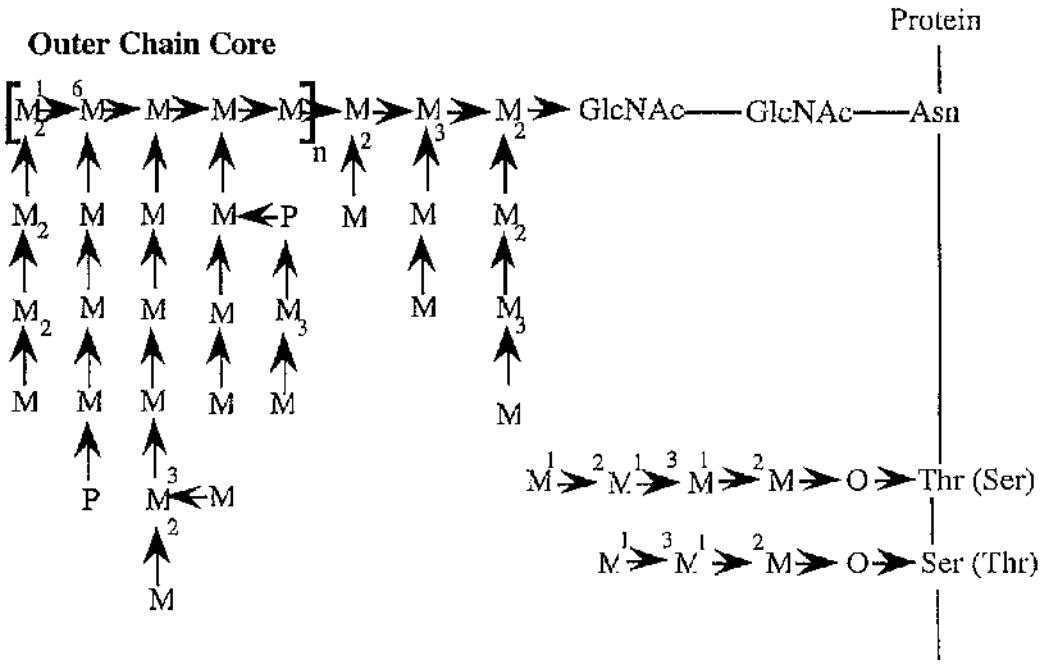


Figure 2. Representation of cell wall mannoprotein of *C. albicans* (based on Shepherd and Gopal, 1991)



the major antigenic sites, an inner core and the base-labile oligosaccharides. Highly branched polysaccharides with N-linkages are attached to asparagine residues of protein through a *N*-acetyl-D-glucosamine (GlcNAc) dimer bridge and a small linear chain with O linkages of mannose residues attached through serine and threonine. The O-linked and N-linked oligosaccharides account for about 10 and 90%, respectively, of the total mannose residues. Mannan estimation has been proposed as a measure of the growth of *C. albicans* (Pike *et al.*, 1991). Details of the structure are presented in Figure 2.

Several catalytic mannoproteins have been detected outside the plasma membrane barrier such as acid phosphatase and beta-glucanase. Other components notably acid proteinase, are believed to be important as virulence factors (Rüchel *et al..*, 1992).

1.2.4.2. Glucans

The glucose polymers, the glucans, are 1) a highly branched β-1, 6 glucan, 2) a highly branched β-1, 3 glucan and 3) a mixed β-1, 3 and β-1, 6 glucan complex with chitin (Shepherd, 1987). Glucans were separated on the basis of their solubility in acid and alkali. They have been classified as alkali-soluble glucan, acetic-soluble glucan and the acid/alkali insoluble glucan. Although the proportion and nature of glucans were similar in yeast cells and hyphae, the alkali-insoluble glucan contained considerably more β-1, 3 linkages during early stages of germ tube formation (Gopal *et al.*, 1984).

1.2.4.3. Chitin

Chitin is a β-1,4 polymer of *N*-acetyl-D-glucosamine and quantitatively is only a minor component of the *Candida* cell wall. It confers rigidity and may play a role in determining the shape of the cell (Hilenski, *et al.*, 1986). Chitin was found with the buds scars and septa (Tronchin *et al.*, 1981). Smaller amounts of chitin are distributed throughout the wall (Braun and Calderone, 1978). Its content in the wall varies with

the growth form, since hyphal-form walls contain roughly three times more chitin than those of yeast cells (Chattaway *et al.*, 1968; Sullivan *et al.*, 1983).

1.2.4.4. Lipids

The lipid composition of *C. albicans* varies considerably, depending on culture age, morphological forms and type of media supplementations (Ghannoum *et al.*, 1986). The cellular lipids of *C. albicans* consist of two major classes of lipids, neutral and phospholipids. Misha and Prasad (1991) have reviewed this topic.

1.2.4.5. Extracellular polymeric material

C. albicans produces an additional fibrillar cell-surface layer after growth to stationary phase in medium containing high concentrations of galactose or sucrose (McCourtie and Douglas, 1981, 1984). These fibrils are mannoproteins and easily released from the cell surface, and extracellular polymeric material (EP) can be isolated from culture supernatants. There is evidence that the degree of development of this fibrillar layer is related to the adherability of the yeast (McCourtie and Douglas, 1981; 1984). The material contains 65-82% carbohydrate (mainly mannose), with smaller amount of protein (7%), phosphorus (0.5%) and 1.5% glucosamine (McCourtie and Douglas, 1981, 1984; Douglas, 1991).

The extracellular polymeric material (EP) from *C. albicans* in yeast nitrogen base medium containing a high concentration galactose inhibited adherence of yeast to buccal epithelial cells (McCourtie and Douglas, 1985). High concentration of sugars in the medium is believed to increase the fibrillar layer and EP of *C. albicans*. It was suggested that EP contains one adhesin which can bind to and block epithelial receptors. The interaction is quite specific, as mannoprotein isolated from *C. albicans* GDH 2023 failed to inhibit the adhesion of strain GDH 2346 (Critchley and Douglas, 1987b). Further characterization of the mannoprotein allowed the identification of the portion of the molecule which participates in the interactions with epithelial cells. Pretreatment of crude adhesin with heat, dithiothreitol, or proteolytic enzymes such as

chymotrypsin, trypsin, pronase (except papain) either partially or completely abolished its ability to inhibit adhesion to buccal cells. Whereas pretreatment of EP with sodium periodate or α-mannosidase had little or no effect (Critchley and Douglas 1987a). Moreover, the protein-rich fraction obtained by incubating crude adhesin with endoglycosidase H inhibited attachment more than did the carbohydrate-rich fraction (Critchley and Douglas 1987a). Adhesin-receptor interactions between yeast and buccal cells seems to be via protein portions of the mannoprotein adhesin. Mannoproteins from crude adhesin preparations were capable of binding to *N*-acetyl D-glucosamine, L-fucose and D-mannose. The lectin-like adhesin on the mannoprotein of *C. albicans* may interact with a glycoside receptor (either glycoprotein or glycolipid) on the host surface. Recently, fucose-binding protein fragments were recovered by affinity adsorption with the trisaccharide determinant of the H (type 2) blood group antigen which terminates in a residue of L-fucose. This purified adhesin, which was devoid of carbohydrate, inhibited yeast adhesion to buccal epithelial cells 221 times more efficiently, on a protein weight basis, than did EP. Adhesion inhibition reached a maximum of 78 to 80 % at an adhesin concentration of 10 μg ml^{-1}. Tosh and Douglas (1992) reported that this protein is the major adhesin of the yeast-form cells of *C. albicans*. Further information about lectin-like adhesins is given in section 1.4.1.1.

1.2.5. Morphogenesis

It was recognised over a century ago the fungus is a pleomorphic fungus (Section 1.1). The organism in fact displays four different cellular forms: yeast cells (blastospores), pseudohyphae, hyphae and chlamydospores. But it is generally called a dimorphic yeast because the transition from the yeast cell to hyphae is an important step in pathogenicity. These different forms depend on the nature of the growth medium *in vitro* (Odds, 1985). Fungal morphogenesis has been reviewed by Odds (1985) and Shepherd (1991).

The yeast cell (blastospore) is a unicellular form produced by a specific process of mitotic cell division known as budding, which involves growth of new cellular material from a small selected side on the yeast cell surface. The new bud enlarges until at a critical stage in its development, mitosis occurs and a septum is laid down at the neck between the parent and daughter cell units. Ultimately the two cell units separate to form two yeast cells. The yeast cell may also give rise to a cylinder-shaped new cellular structure, the germ tube, which grows continuously by apical extension. Further elongation of the germ tube gives rise to a hypha although there is lack of agreement about the point at which the name should change. According to Odds (1988), the name "germ tube" should be used only to refer to the newly evaginating hypha up to the time of formation of the first septum. Thus hyphae contain multiple fungal cell units divided by septa. Mitotic cell division occurs within the extending hypha, and septa are formed at intervals along its length without interruption of the rate of extension. The hyphae, in turn, may give rise to new yeast cells that bud off laterally or may produce hyphal branches.

Pseudohyphae (Mackenzie, 1964) arise from yeast cells or from hyphae by a budding process. They are essentially elongated yeast cells attached to each other with constrictions and sometimes giving the appearance of a filamentous cell chain gives a gross appearance similar to true hyphae They are microscopically distinguishable from true hyphae only by the conspicuous constrictions at septal junctions. Chlamydospores (Benham, 1931) are large, refractile, thick-walled asexual spores and are only produced by *C. albicans in vitro* .

1.2.6. Candidosis

Candidosis is a spectrum of infections that may present themselves as superficial, cutaneous, mucosal, systemic or disseminated. Full accounts are presented by Odds, (1988) and Bodey who is editor of the book (1993) from which the following summary has been prepared.

1.2.6.1. Predisposing factors

There are a number of factors that increase the likelihood of *Candida* infection. In general, they alter the balance between the host and the normal commensal flora, i.e. the excessive growth of the fungus or breakdown of the normal physical and immunological defences of the host may render *Candida* species pathogenic. *Candida* infection is influenced by age of the host, i.e. neonates or elderly individuals being more often associated with candidosis. Vaginal carriage of yeast is greater in pregnant than in nonpregnant women. Dietary factors such as a carbohydrate-rich diet, or vitamins, are factors predisposing to candidosis. Mechanical factors and trauma such as burns, surgery, catheters or drug abuse may place the patient at risk for developing candidemia from a cutaneous source. Candidosis may be found in patients with underlying diseases such as diabetes mellitus, cancer and various infections, notably HIV (Anaissie, 1992; Coleman *et al.*, 1993). The use of some antibiotics and chemotherapy are also factors for developing deep-seated *Candida* infection (Karabinis *et al.*, 1988; Kennedy and Voltz, 1985).

1.2.6.2. Superficial candidosis

The most common *Candida* infection is superficial candidosis, characterized by infections to lining surfaces such as the gastrointestinal tract skin, oropharynx and upper and lower respiratory tracts. The gastrointestinal tract is usually believed to be the major source of *Candida* species.

Cutaneous candidosis has been claimed to be generally more common among females than males. The infection is more common among white-skinned than black-skinned individuals. *Candida* intertrigo may be seen in association with many kinds of underlying disease. The most significant factor predisposing to the condition is wetting of the skin. *C. albicans* does not survive well on dry skin, but can survive for very long periods when skin is kept moistened. Nail and nailfold infection with *Candida* have been described particularly in housewives. The fingernail infections are more common than those of toenails .

Chronic Mucocutaneous Candidosis (CMC) is a form in which superficial candidosis most commonly affects the mouth or vagina; infections remain limited to these surface body sites and can be treated successfully. *C. albicans* penetrates only as far as the stratum corneum. The affected tissues also contain infiltrations of monocytes, lymphocytes and polymorphonuclear neutrophils. Accumulation of inflammatory cells at infected sites suggests that the host is able to mobilize cellular defenses against *Candida,* but unable to eliminate the pathogen totally. Failure of the host to eradicate the fungus appears to be related in some way to defective cell-mediated immunity commonly associated with CMC.

Oral candidosis is a well-known disease. Various *Candida* species can cause thrush and denture stomatitis. The thrush, defined as clinical oral candidosis (creamy-white, curd-like patches on the tongue or other oral mucosal surfaces) may extend to the oesophageal mucosae to produce oesophagitis. Oral thrush is most prevalent in infancy and old age and among the terminally ill (Roseff and Sugar, 1993). In denture stomatitis a link has been found between *Candida* and denture-related palatal inflamation. It is very common in patients who wear dentures, because *C. albicans* adheres well to the plastics used in these appliances (Samaranayake *et al.*, 1980). The inflammation in denture stomatitis seems to arise mainly in response to the growth of large numbers of yeast and hyphae of *Candida* in the space between dentures and the palatal epithelia (Davenport, 1970).

Candida infections of the female genitalia are approximately ten times more common than infections of the penis. Vaginal thrush has been noted in women with pregnancy or diabetes. A higher prevalence of genital candidosis has been seen among women of low socioeconomic status. Candidosis of the genitalia can occur by sexual transmission.

1.2.6.3. Systemic candidosis

The most severe type of *Candida* infection is deep or systemic candidosis recognizable by intraparenchymal lesions usually involving the heart, kidney, liver,

spleen, lung and brain. Systemic candidosis is characterized by formation of multiple abscesses. It may cause disease in patients who have been immunosuppressed by malignancies or postoperatively.

Systemic forms of candidosis may arise as localized primary diseases, with only one organ affected, or as disseminated candidosis (*Candida* - septicaemia) in which *Candida* spreads by the blood stream to invade several organs. There are difficulties in diagnosis of systemic candidosis. One of them is the isolation of *C. albicans* from infected tissues. Isolation of *Candida* from blood cultures sometimes gives negative results, even in the otherwise proven cases of deep candidosis. Sometimes positive results may be dismissed as accidental contamination. Serological tests are most effective when blood samples are collected at regular intervals.

The gastrointestinal tract may be infected by *C. albicans* from its mucosal surface or via the bloodstream. Gastrointestinal candidosis can be diagnosed by histological proof of candidal tissue invasion in biopsy material from a lesion.

1.2.6.4. Disseminated candidosis

Disseminated candidosis arises most often in patients who have been hospitalized and immunocompromised for relatively long periods. The mortality rate associated with cases of disseminated candidosis is high. Malignant diseases and the gastrointestinal tract are the primary factors for disseminated candidosis; and surgery, chemotherapy with multiple antibiotics, cytotoxic drugs and corticosteroids, and the use of indwelling intravascular catheters are major iatrogenic contributors to the infection.

1.2.6.5. Treatment

Candida spp. are eukaryotic cells, like human cells, and not prokaryotic like bacteria; hence, antifungal agents are associated with substantially more toxicity than antibacterial agents. Reviews on antifungal drugs may be found in (Shepherd, 1985; Odds, 1988). What follows below is no more than a brief outline of a large subject.

The variety of antifungals which are currently used in treatment of candidosis are categorized into two major groups: polyenes and azoles. Polyene antifungals (Amphotericin B, Candicidin, Nystatin) are producted by Streptomyces and are various substituted derivatives of the natural compounds. All polyenes damage membranes in eukaryotic cells and alter their permeability. Some of them exhibit a degree of selective toxicity for fungal membranes. The membrane-damaging action of polyenes is due to their high avidity for binding to sterols, particularly ergosterol. Some investigators (Al-Bassam *et al.*, 1985) observed that the level of cell-wall mannan content of *C. albicans* can change after polyene treatment. In addition, the stationary-phase yeast cells were more resistant to the permeabilizing action of polyenes than exponential phase yeast cells. Polyenes seem to give lower anti-*C. albicans* MIC's in conditions that favour growth of the fungus in the hyphal rather than the yeast form. However, polyenes do not have a specific effect on the morphological development of *C. albicans*. Under laboratory conditions, many isolates of *Candida* species can be induced to develop resistance to polyene antifungals. The change in sterol composition and cell wall permeability are responsible for this resistance.

Amphotericin B, produced by the actinomycete *Streptomyces nodosus*, has been used for systemic and superficial treatment but is toxic to humans. A similar agent, Nystatin, is restricted to topical use only because of its toxicity and insolubility. Nevertheless it is valuable for oral and vaginal forms of candidosis. Candicidin is too toxic for systemic chemotherapy. It has been used for treatment of vaginal candidosis. Natamycin is more effective than other topical polyenes and can be used for vaginal candidosis. Trichomycin has been used successfully in several trials in vaginal candidosis, but has not been introduced for routine clinical use. It is too toxic for systemic application in humans.

Azole antifungals: Several azole compounds are available for the treatment of *Candida* infections, including miconazole, ketoconazole, fluconazole, and itraconazole. All the antifungal azoles cause alterations in the sterol composition of

Candida membranes by their action on cytochrome (Vanden Bossche *et al.*, 1980). Most of them can exert fungicidal effects by directly damaging the membrane of *Candida* cells. Most azoles are able to prevent hyphal growth and branching and have been used in studies on hyphal virulence (Johnson *et al.*, 1983; Odds, 1985; Ryley and Ryley, 1990). Since the hyphal form of *C. albicans* may be important in some aspects of pathogenesis, it can be theorized that the effects of azoles on morphogenesis are important in preventing development of invasive candidosis in mammalian hosts (Odds, 1985; Ryley and Ryley, 1990).

Flucytosine: Many systemic forms of *Candida* infection have responded to flucytosine treatment which is less toxic than amphotericin B. But there is a problem of resistance to the drug. Because of this problem, use of flucytosine is indicated only in serious, life-threatening yeast infections (Odds, 1988) .

1.3. PATHOGENIC *CANDIDA* SPECIES

Candida is unique among opportunistic pathogens because it is a ubiquitous fungus commonly found in the human and animal flora of the mouth, oropharynx, intestines, vagina and skin. Although numerous species of *Candida* are described in the microbial cosmos, human infection mostly is caused by *C. albicans* and *C. tropicalis* of which the former is by far the most adhesive species (McCourtie and Douglas 1984). The other species isolated as cause of disease in humans are *C. glabrata*, *C. paraspilosis*, *C. guilliermondii*, *C. rugosa* and *C. krusei*. The various species of *Candida* are described in detail by Odds (1988) and Bodey (1993).

1.4. *CANDIDA* VIRULENCE

There are numerous microbial and fost factor which influence the virulence of *C. albicans* (Table 1). These are discussed principally within the context of adhesin-receptor interactions in the following section.

1.4.1. Adhesin-Receptor Interactions

C. albicans has multiple mechanisms for its interactions with host cells. These interactions have been categorized into 5 types (Table 2); lectin-like, protein-protein and mannan, lipid and chitin interactions, as reviewed by Hostetter (1994a) and Odds (1994).

1.4.1.1. Lectin-like interactions

Binding of bacteria to host cells is inhibited by simple sugars, indicating these activities are due to lectins present on microorganisms, as first suggested by Ofek *et al.* (1977) and Ofek and Beachey (1978) for *Escherichia coli.* The receptor molecules that carry the carbohydrate structures complementary to the bacterial lectins may be glycoproteins and/or glycolipids (Rauvala and Fine, 1979).

Lectin-like interactions in the present context are those in which a protein on the candidal surface recognizes a carbohydrate on the epithelial surface. Sandin *et al.* (1982) inhibited candidal binding to buccal epithelial cells by adding α-methyl-mannoside to the assay mixture. Other investigators reported that neither mannose nor α-methyl-mannoside could directly reduce adherence of the yeast cells to vaginal epithelial cells (Sobel *et al.* , 1981; Segal *et al.* , 1982a; Lee and King, 1983). Fucose, however, inhibited adhesion of *C. albicans* in a similar system (Sobel *et al.*, 1981). The study by Segal *et al.* (1982a) showed that amino sugars partially blocked *C. albicans* adherence. Centeno *et al.* (1983) found that mannose inhibited attachment

Table 1. Factors affecting the virulence *C. albicans* (based on Olsen, 1990)

Factors related to yeast cells
Medium / cultivation
Phenotypic switching
Germ tube, hyphae production
Hydrophobicity
Enzymes
Cell wall components, (mannoprotein, mannan, chitin, lipids, extracellular polymeric material)
Serotype A
Factors related to host cells
Cell source
Mucosal cell size and viability
Extracellullar matrix proteins
Fibrin
Sex hormones
Donor antigen, ABO[H]
Factors related to the environment
Cations
pH
Sugar
Saliva
Humoral antibody and serum
Bacteria
Antibacterial drugs
Drugs
Lectins

Table 2. *C. albicans* - host cell interactions (based on Calderone *et al.*, 1994)

System	*C. albicans* factor	Host cell factor	References
I	Mannoprotein [a] lectin (s)	Fucose/NAGA glycosides	Critchley and Douglas, 1987a, b; Douglas, 1987a,b, 1992; Tosh and Douglas, 1992.
II	CR2/CR3-like mannoprotein[a]	RGD-containing ligands	Alaei *et al.*, 1993; Boluali *et al.*, 1986; Bouchara *et al.*, 1990; Gilmore and Hostetter, 1986; Gilmore *et al.*, 1988; Gustafson *et al.*, 1991; Klotz and Smith, 1990; 1991; Klotz *et al.*, 1992; Ollert *et al.*, 1990.
III	Mannan factor 6	Unknown	Miyakawa *et al.*, 1992.
IV	Chitin	Unknown	Lehrer *et al.*, 1988.
V	Mannoprotein[a]	Mannose receptor	Li and Cutler, 1993.

System I, III, IV- Epithelial cells

System II-Endothelial cells

System V-Splenic macrophages

[a]The underlined component indicates the ligand binding domain.

of *C. albicans* to human urothelial epithelial cells when sugar was added to the incubation solutions. But glucose had no significant effect on yeast cells attachment. Soon afterwards, a crude mannoprotein preparation from yeasts grown in 500 mM galactose was shown to inhibit adhesion of *C. albicans* to buccal epithelial cells (McCourtie and Douglas, 1985). Moreover, treatment of these crude extracts with heat, dithiothreitol or several proteolytic enzymes other than papain abrogated their inhibitory activity, whereas treatment with sodium periodate, α-mannosidase, or endoglycosidase H was ineffective (Critchley and Douglas, 1987a). Addition of L-fucose to an *in vitro* adhesion assay with *C. albicans* strain GDH 2346 inhibited yeast attachment to buccal epithelial cells. Pretreatment of these cells with lectin from *Lotus tetragonolobus* (which is specific for L-fucose) but not other lectins blocked adhesion (Critchley and Douglas, 1987b). By contrast, adhesion of strain GDH 2023 was inhibited by *N* -acetyl-D-glucosamine and wheat germ tube agglutinin (a lectin specific for *N* -acetyl-D-glucosamine) but not by L-fucose or *L. tetragonolobus* lectin. These results indicated that *C. albicans* has strain-specific adhesins. Critchley and Douglas concluded that the protein portion of the mannoprotein is responsible for receptor recognition. Thus the adhesins of this organism, which recognize sugar-containing receptors of epithelial cells were correctly identified as lectins (Douglas, 1992; Calderone and Wadsworth, 1993).

This type of interaction is represented by candidal mannoproteins, which recognize a variety of carbohydrates on blood group O cells (Burford-Mason *et al.*, 1988; Thom *et al.*, 1989). Hosts with blood group O express on their buccal and vaginal epithelial cells H antigen, a glycoside containing D-galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine and an α-1, 2 fucose moiety. The interaction of lectin-like mannoproteins on *C. albicans* and fucosylated antigens on buccal and vaginal epithelial cells could explain the well-known predilection of nonsecretors and hosts with blood group O for oral and vaginal candidosis (Thom *et al.*, 1989). Binding of *C. albicans* to non-secretor epithelial cells was inhibited by pretreating the cells

with anti-Lewis^a. This result suggested that Lewis^a might be one of the receptors for some yeast cells.

In contrast, Brassart *et al.* (1991) reported that Lewis^a antigens failed to act as adhesin inhibitors. There is some evidence that the adherence of *C. albicans* to Lewis^a antigen is strain dependent (May *et al.*, 1989). Human milk oligosaccharides, containing the Fuc α1-->2 Gal β determinant, (the H-sugar sequence found on all blood group substance of the ABO[H] system) blocked adherence of *C. albicans* to human buccal epithelial cells (Brassart *et al.* ,1991). As concluded by Hostetter (1994a), the H and Lewis^a antigens would seem to be prime candidates for fucose-containing epithelial cell receptors.

The coupling of blood group determinants Lewis^a, Lewis^b, and H to an affinity column permitted the characterization of a presumed mannoprotein adhesin from *C. albicans* GDH 2346 (Tosh and Douglas, 1992). Fucoside-binding fragments were then recovered by affinity adsorption with the trisaccharide determinant of the H (type 2) blood group antigen which terminates in a residue of L-fucose. Although the mass of this protein was not cited, the purified protein, devoid of carbohydrate, inhibited to a maximum of 78 to 80 % at an adhesin concentration of 10 μg ml^{-1}. It was 221 times more active than the crude extract in inhibiting adhesion to buccal epithelial cells. These results indicated that this protein is the major adhesin of yeast-phase cells of *C. albicans* GDH 2346 but that one or more secondary adhesion mechanisms may also act (Tosh and Douglas, 1992).

Jimenez-Lucho *et al.* (1990) found that *C. albicans* bound the glyco-sphingolipid, lactosylceramide, which does not contain L-fucose. Recently, Cameron and Douglas (1996) found that extracellular polymeric material of *C. albicans* GDH 2346 bound to L-fucose-containing glycosphingolipids from human BEC and sheep red blood cells.

As reviewed by Calderone (1993), several investigators observed that while yeast adhesion to epithelial cells involves lectin-like adhesins to a glycoside receptor, lectin-like adhesins on germ tubes or hyphal form remain to be clarified.

1.4.1.2. Interactions with host proteins

Protein-protein interactions are those in which a protein on the candidal surface recognizes a protein or peptide ligand on epithelial or endothelial cells.

1.4.1.2.1. *Fibronectin* Initial studies of endothelial adhesion suggested a prominent role for fibronectin as a ligand for *C. albicans* and *C. tropicalis* which adhered to fibronectin in cell-culture wells (Segal *et al.*, 1982b; Scheld *et al.*, 1981; 1985; Skerl *et al.*, 1984). Because the yeasts most frequently involved in endocarditis were also the most adherent *in vitro,* the fibronectin on the vascular surface might mediate microbial adhesion in this disease. It was also suggested that free fibronectin in serum could occupy fibronectin adhesins on yeast cells and prevent their attachment to fibronectin in the endothelial matrix.

Skerl *et al.* (1984) deposited radiolabelled yeast cells in fibronectin-coated microtiter wells. By counting the radioactivity in the washes of the wells, the investigators determined that approximately 30 to 40% of *C. albicans* cells adhered in a calcium-dependent fashion; adhesion of *C. tropicalis* was slightly greater when tested side by side with *C. albicans* , but *C. krusei* and *C. pseudotropicalis* failed to adhere to fibronectin. Pretreatment of yeast cells with fibronectin decreased adhesion to buccal or vaginal epithelial cells by 40 to 50 %. The authors concluded that surface proteins of *C. albicans* and *C. tropicalis* mediated adhesion of yeast cells to fibronectin on epithelial surfaces (Skerl *et al.* , 1984).

Klotz (1987) showed adhesion of *C. albicans* and *C. tropicalis* to subendothelial matrix proteins, as opposed to endothelium itself. *C. albicans* and *C. tropicalis* adhered more avidly to the contracted monolayer, which exposed subendothelial extracellular matrix, than to a confluent monolayer of bovine aortic endothelial cells (Klotz and Maca, 1988). Binding of fibronectin by one of four clinical isolates of *C. albicans* was shown to be saturable, specific and reversible. Purified fibronectin possesses two peptide-form RGD sites in the fibronectin cell-

binding domain, RGD and GRGDTP and a control peptide GRGESP. All of these inhibited the binding of fluid-phase fibronectin to *C. albicans,* whereas carbohydrates (including D-glucose, α-methly-D-mannopyrannoside and D-mannose did not (Klotz and Smith, 1991).

The fungus also possesses a detergent-extractable, surface glycoprotein adhesins (Klotz *et al.*, 1993). These adhesins were capable of adhering to immobilized plasma protein, fibronectin, in the presence of calcium, and also was eluted from fibronectin-agarose affinity columns with EDTA. Klotz *et al.* (1994) found a glycoprotein on *C. albicans* from a fibronectin-agarose affinity column using high-performance liquid chromatography. The adhesin consisted of approximately 75 to 80 % carbohydrate and 20-25% protein by weight and existed as an aggregate or multimer *in vitro* (Klotz *et al.*, 1994).

1.4.1.2.2. ***Fibrinogen*** The distribution of fibrinogen-binding protein was reported to be primarily on the germ tubes portion of the hyphal form, with occasional expression by emerging buds, and generally homogeneous on young hyphae, as determined by indirect immunofluorescence (Bouali *et al.*, 1986, 1987; Page and Odds, 1988). The binding of fibrinogen on the cells was observed by scanning and transmission electron microscopy after the cells had been incubated with fibrinogen-coated latex microspheres or gold particles (Bouali *et al.*, 1986; Tronchin *et al.*, 1987). Fibrinogen-coated gold particles were associated with the surface layer of germ tubes. Fibrinogen immobilized on various supports such as plastic dishes, polystyrene microspheres or dextran macrobeads gave similar results, showing the binding of *C. albicans* germ tubes to fibrinogen-coated supports (Page and Odds, 1988; Tronchin *et al.*, 1988; Bouchara *et al.*, 1990). Fibrinogen binding was quantitated for both yeast and hyphal forms (Bouali *et al.*, 1987). Binding was 12-fold higher for hyphae and 7.7-fold higher for germ tubes than for yeast forms.

The interaction between human fibrinogen and *C. albicans* germ tubes was characterized using ^{125}I-labelled fibrinogen and fragment D. The binding was found

to be time-dependent, saturable, reversible and specific, hence demonstrating the characteristics of true receptors. Analysis of data by the Scatchard equation revealed that there were about 6,000 binding sites per germ tube, with a dissociation constant of 5.2×10^{-8} M, indicative of a strong affinity of fibrinogen for *C. albicans* (Annaix *et al.*, 1990). In another study, mice were injected intravenously with various strains of viable *C. albicans*. Four day later, the animals were sacrificed and thin sections of kidney were examined by immunofluorescence microscopy with an anti-fibrinogen antiserum. In contrast to what was observed *in vitro*, both yeast cell and germ tube portions of the hyphal-form cells were found to be surrounded by a thick coat of fibrinogen *in vivo*. This finding may be related to the cells being different when grown *in vivo* compared to *in vitro* (Robert *et al.*, 1991a). Fibrinogen binding adhesins on yeast and germ tube portion of the hyphal form cells was recently confirmed by Martinez *et al.* (1994),

1.4.1.2.3. ***Complement*** The first observation of the complement C3 conversion product, (C3d) receptor on *C. albicans* was made by Heidenreich and Dierich (1985). Later, it was confirmed by several investigators (Gilmore *et al.*, 1988; Gustafson *et al*, 1991; Alaei *et al*, 1993). As reported by Heidenreich and Dierich (1985), C3d-coated sheep erythrocytes (EAC3d) form rosettes with hyphal form *C. albicans*. Solubilized mannan, D-galactose, L-mannose and *N*-acetyl D-glucosamine failed to inhibit binding of the hyphal form. Other sugars such as D-glucose and D-mannose did block the binding to hyphae by approximately 30%. Killing the fungus by heat or treatment with proteases such as trypsin or pronase, resulted in binding activity being significantly blocked, and suggested that the receptor may be a protein. According to Gilmore *et al.* (1988), iC3b receptors are present on both yeast and pseudohyphal forms. Hyphal-form cells possess two- or three-fold more receptors on their surfaces and were less taken up by human polymorphonuclear leukocytes than yeast forms, because of the function of iC3b receptor which might impair phagocytosis. They also observed that growth of *C. albicans* in the presence of 50 mM glucose increased

binding of iC3b, compared with growth in 5 mM D-glucose. When an avirulent mutant strain was compared with its parent after production of hyphal-form cells, iC3b receptor was found on the parent strain , this receptor might therefore be a virulence factor of the fungus.

1.4.1.2.4. ***Laminin, vitronectin and others*** Laminin a major component of the mammalian basement membrane, is a large multidomain glycoprotein which seems to play a critical role not only in normal cell adhesion but also in interaction with microorganisms. The presence of laminin receptors on *C. albicans* germ tubes has been reported by Bouchara *et al.* (1990), But the yeast cells did not interact with soluble laminin. Recently, laminin-binding sites on yeast cells have been identified (Klotz *et al* ., 1992; Lopez-Ribot *et al.*, 1994). Binding of the yeast and hyphal-forms *C. albicans* to entactin was reported by Lopez-Ribot and Chaffin (1994). The entactin-binding material (s) in the fungus cell wall also displayed some ability to bind fibronectin and laminin. Human albumin and transferrin bound with high avidity to the germ tube portion of the hyphal-form cells (Page and Odds, 1988).

Vitronectin, is a 75kD glycoprotein, with multiple regions which promote cell adhesion and proliferation. Binding of vitronectin to *C. albicans* has been documented by Jakap *et al.* (1993) and by Limper and Standing (1994).

1.4.1.3. Mannan

Mannan is a possible adhesin of *C. albicans* (Kanbe *et al.*, 1993). The serotype-A specific chain of cell wall mannan, designated as antigen 6, was mainly involved as a ligand in adherence of *C. albicans* A to epithelial cells. (Mikayawa *et al.*, 1992). These authors prepared a monoclonal antibody (MAb-6) which recognized oligosaccharides of the cell wall mannan specific for serotype A, and used it in screening for antigen-6-deficient mutants (Mikayawa *et al.*, 1986). The mutant strains exhibited reduced adherence to buccal epithelial cells in suspension when compared with the parent strain. The role of antigen 6 in adherence of *C. albicans* serotype A to

keratinocytes have been examined by Tsuboi *et al.* (1994). A cultured keratinocyte monolayer was used to simulate conditions encountered *in vivo* between the organism and epithelial cells. A parent strain and its antigen-6-deficient mutant strain were used. A yeast form of *C. albicans* was inoculated onto monolayers of normal human keratinocytes, and adherent organisms were assessed semi-quantitatively by scanning electron microscopy. The number of adherent organisms was signifcantly lower for the mutant strain than for the parent strain. These results suggested that antigen 6, located on the cell wall, has an important role in the initial stage of the adherence of *C. albicans* A to keratinocytes.

1.4.1.4. Lipid

Experimental evidence for the involvement of lipids in adherence of *Candida* species to BEC has been provided by Ghannoum *et al.* (1986). Subsequent experiments have shown that total lipid extract of cell wall and from whole epithelial cells blocked adherence (Ghannoum *et al.,* 1987).

1.4.1.5. Chitin

Chitin is available on the cell wall in smaller amount than other cell wall components and may act as an adhesin of human vaginal epithelial cells (Segal *et al.*, 1982a, 1988) and intestinal mucosa *in vitro* (Segal and Savage, 1986). Inhibition experiments with a chitin-soluble extract showed that a cerulenin-resistant mutant of *C. albicans* adhered less to human vaginal mucosal cells *in vitro* than did a wild type (Lehrer, *et al.*, 1986). They also showed that the mutant strain was less virulent in the murine model of vaginal candidosis.

1.4.2. Toxins

Candida toxins, analogous to bacterial exotoxins and endotoxins, have been sought but none with biological properties equivalent to those of bacteria have so far been found (Nelson *et al.*, 1991). *C. albicans* endotoxin was suggested but not isolated by

Hansenclever and Mitchell (1963). Glycoprotein extracts of *Candida* cell wall, resemble bacterial endotoxins in being lethal and pyrogenic and able to induce anaphylactic shock in various animal models (Cutler *et al.*, 1972). But their potencies are not comparable to the bacterial materials. Failure to identify a potent candidal exotoxin may not be suprising, however, since a microorganism in frequent commensal contact with humans is unlikely to produce a potent exotoxin and retain its status as an opportunistic pathogen (Odds, 1988).

1.4.3. Enzymes

The extracellular proteolytic activity of *C. albicans* was discovered by Staib (1965). The species possesses a family of aspartic (acid) proteinases at least one of which is secreted extracellularly in an active form. The secreted acid proteinase (SAP) of *C. albicans* is a 41.5 kDa protein and capable of degrading epidermal keratins, denatured type IV collagen, laminin, fibronectin, albumin, hemoglobin, immunoglobulin heavy chains and other proteins over a broad acid pH range (Tsuboi *et al.*, 1994). Budtz-Joergensen (1971) first observed an association between proteolytic *C. albicans* and mucosal disease (denture stomatits). MacDonald and Odds (1980) confirmed, by immunofluorescence, the induction of *Candida* proteinase during murine candidosis of the kidney. High titers of specific antibodies were monitored in sera of patients suffering from candidosis (MacDonald and Odds, 1980). The investigators showed that the enzymes were expressed in infected tissues *in vivo*. Some SAP-deficient mutant strains of *C. albicans* were less virulent in mice than the strongly proteolytic parental strain (MacDonald and Odds, 1983). This was confirmed by Kwon-Chung *et al.*, (1985) who showed also that revertants with restored proteolytic activity regained their virulence. A proteinase-deficient mutant was less mouse-lethal than its parent strain. Secretion of SAP correlated with hyphal invasion of the chick chorioallantoic membrane (Shimizu *et al.*, 1987). In experimental murine cutaneous candidiasis models, pathogenic-SAP sufficient strains produced cavitations in the surface of skin corneocytes around the adherent yeast cells

in a time-dependent manner (Ray and Payne, 1988). Ghannoum and Abu-Elteen (1986) observed that *C. albicans* isolates which adhered most strongly to BEC had the highest relative proteinase activities and were pathogenic. Evidence for a role of *Candida* proteinase in the pathogenesis of candidosis was also reported by Rüchel *et al.*. (1992).

A phospholipase activity has been correlated with mouse lethality and adherence of fungal cells to buccal epithelium (Barrett-Bee *et al.*, 1985). It was also supposed that the germ tube or hyphal form of the organism directly penetrates to the epithelial cell membrane, possibly aided by a phospholipase or other hydrolytic enzyme (Pugh and Cawson, 1977).

1.4.4. Phenotypic Switching

Most strains of *C. albicans* are capable of switching between a number of general phenotypes which can be discriminated by colony morphology. Phenotype switching was first noted for *C. albicans in vitro* (Slutsky *et al.*, 1985). Subsequently, studies have linked switching *in vitro* with virulence *in vivo* (Soll, 1988). Switching is reversible and can affect most physiological and morphological characteristics of the cell, including many of the putative virulence factors (Soll, 1992.) Changes in adhesion due to switching were first observed in the white-opaque phase transition in strain WO-1 Kennedy *et al.* (1988). Cells in the white phenotype showed greater adherence than cells in the opaque phenotype to buccal epithelium, but cells in the opaque phase exhibited a higher level of coadhesion (Kennedy *et al.*, 1988). Phenotypic switching of *C. albicans* was reviewed by Soll (1992).

1.4.5. Dimorphism

Dimorphism, associated with increased virulence, was observed by Hill and Gerbhardt (1956), Young (1958) and Kozinn *et al.* (1960). *C. albicans* was found to adhere better to epithelial cells under conditions that enhanced germ tube formation (Kimura and Pearsall, 1980; Sobel *et al.*, 1984). *C. albicans* yeast cells are ingested

in vitro by phagocytic leukocytes and killed, whereas hyphal-form cells are resistent to these processes (Odds, 1988).

1.4.5.1. Production of germ tubes

The yeast-hypha transition in *C. albicans* is controlled by environmental conditions in which the important variables are the carbon source, pH value, ions and temperature. The production of germ tubes in serum has remained the method of choice for identifiying *C. albicans* (Taschdjian *et al.*, 1960).

Several environmental factor are important as stimulators of the morphological transition from yeast to hyphae. However, for any given medium it is widely agreed that incubation temperature and pH and the metabolic state of the cells have critical influence on the morphology.

Germ tubes are induced when yeasts are incubated at temperatures between 33 and 42°C in a medium containing aminoacids such as proline, glutamine and arginine, and aminosugars such as *N*-acetylglucosamine and *N*-acetylmannosamine (Simonetti, *et al.*, 1974; Sullivan and Shepherd, 1982). Furthermore, *N*-acetylhexose derivatives, such as chitin, mucin, hyaluronic acid and immobilized *N*-acetylglucosamine are capable of inducing germ tubes (Shepherd and Sullivan, 1983). Serum, serum derivatives and tissue culture media are also promoters of germ tubes production. These derivatives are not metabolized or transported into the cells; it is believed that the inducer binds to a cell-surface receptor and produces an intracellular signal, which primes the cells for germ-tube formation. Divalent cations, in particular magnesium, are also necessary for germ tube formation. (Walker *et al.*, 1984). Glucose-starved cells inoculated into cultures with added calcium ions grew as hyphal forms while removal of the calcium from the cultures caused the morphology to revert to the yeast form (Holmes *et al.*, 1991). Calmodulin inhibitor (trifluoperazine) has been shown to inhibit the yeast to mycelial transition (Roy and Datta, 1987).

It has been demostrated that actively growing cultures need first to be starved before they produce germ tubes (Shepherd *et al.*, 1980). A pH value in the range 6 - 8

is critical for germ tube formation (Odds, 1988). When yeast cells which have entered the stationary phase of growth in modified Lee medium without aminoacids are transferred into 199 medium at 37°C (Tronchin *et al.*, 1988), they synchronously form buds at pH 4.0 at 37°C and pH 6.7 at 22°C, while germ tubes are formed at pH 6.7 at 37°C as shown in Figure 3 (Tronchin *et al.*, 1988; Lopez-Ribot *et al.*, 1991).

1.4.5.2. Structural analysis of dimorphism

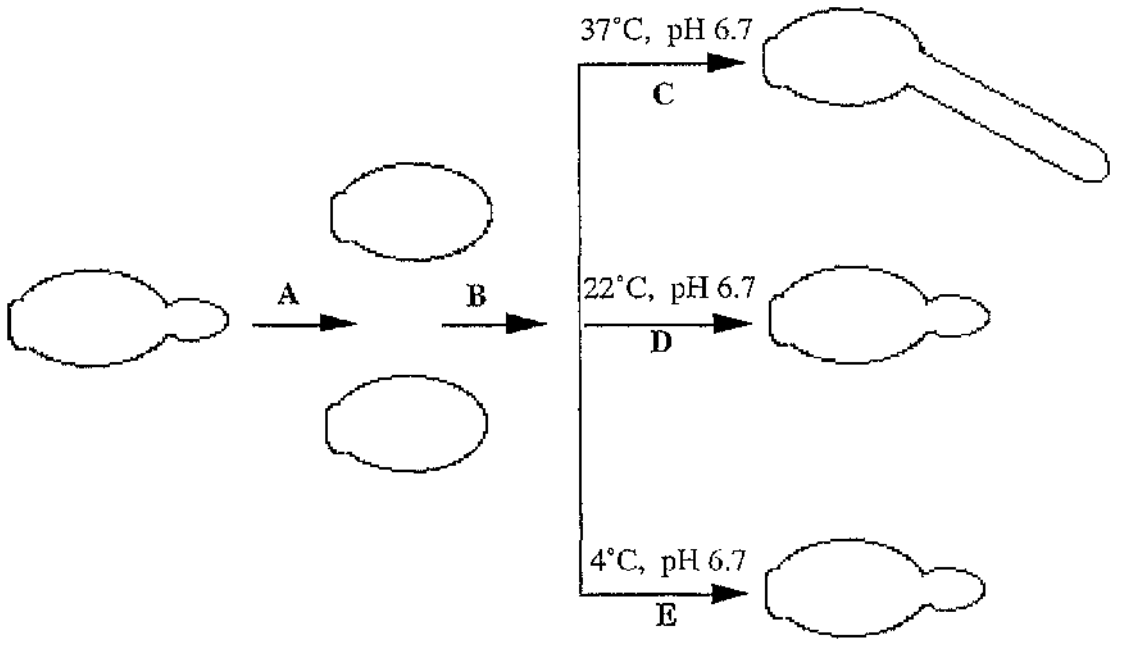
The central observation in *C. albicans* dimorphism is the alteration in cell shape. This difference in cell structure extends to the architecture of the cell wall where changes are evident in transmission electron micrographs (Hubbard *et al.*, 1985). The mycelial cell wall is thinner and the outer layer is more fibrillar than the outer layer of yeast cells. Although the chemical composition of cell walls from yeasts and mycelia are similar (Gopal *et al.*, 1984; Sullivan *et al.*, 1983), the nature of the glucan linkages is markedly different in wall material from cells undergoing the initial stages of hyphal production (germ tubes) which was mentioned in sections 1.2.4.2 and 1.2.4.3. This suggests that as a germ tube emerges, the newly synthesized wall material is markedly different fom yeast wall material and is subsequently modified. These finding have led to the hypothesis that the spatial and temporal control of wall biosynthesis is critical in determining cellular morphology.

1.4.5.3. Practical approaches to studying dimorphism

There are two basic approaches to the study of morphogenesis in *C. albicans*. One is to compare morphological mutants with wild-type parental strains grown under conditions inducing morphogenesis. The other is to compare cells of a particular strain grown under conditions promoting yeast growth with cells grown under conditions promoting mycelial growth.

There are few reports of *C. albicans* morphological mutants and many of those reported had been subjected to extensive mutagenesis. The relevance of morphology to virulence can be understood by studying the capacity of morphology

Figure 3. Experimental systems for stimulating *C. albicans* dimorphism by pH and temperature changes are shown below. Yeast cells from Sabouraud agar (**A**) are grown in Modified Lee Medium without amino acids to starvation at 25°C for 36 h in stationary phase. Yeast cells may require starvation for high yields of germ tubes (**B**). These cells are then released into 199 medium at 37°C and pH 4.0 or 22°C and pH 6.7 (**D, E**) to produce yeast cells. The starved cells are incubated in 199 Medium pH 6.7 at 37 °C (**C**) to produce germ tubes, (based on Shepherd *et al.*, 1985)



mutants to induce experimental infections. A spontaneous mutant, which did not form germ tubes was reported to be less pathogenic compared with its parent strain (Sobel *et al.*, 1984). The other mutant, a nitrosoguanidine-induced morphology mutant of *C. albicans,* was found to be nonpathogenic by Hubbard *et al.* (1986), but pathogenic by another investigator (Shepherd, 1985). Thus the conclusions are unclear. Cannon (1986) described a deficient mutant, MM2002, derived from a parental strain (MEN) by a physical separation method which was straightforward and a simple selection procedure without mutagenesis. Here the stationary phase yeasts of a given strain (MEN) were allowed to produce germ tube and hyphal form cells which were removed by filtration. Then yeast form yeast were collected, grown to stationary stage, placed in conditions to produce germ tubes and yeast forms again physically selected. This process was repeated several times until the isolated yeast had become stabilized and lost ability to produce germ tubes. The incubation of cells in defined medium (pH 6.7) at 37°C induced MEN to grow as mycelia and MM2002 to grow as yeast. While the method is simple, some morphological mutants obtained this way gave rise to revertants (Stewart *et al.* , 1989; Cutler, 1991).

In the absence of appropriate mutants, many workers have studied a single strain under different growth conditions to induce either yeast or mycelial growth. However, in this approach it is of utmost importance to distinguish between morphology-specific and inducer-specific effects. It is possible to compare different media with different carbon sources, different pHs or different temperatures (Odds 1985).

1.4.6. Hydrophobicity

Hydrophobic interactions have been implicated as playing important roles in both adhesion to epithelial cells (Kennedy *et al.*, 1988; Hazen, 1989) and to acrylic (Minagi *et al.*, 1985; Miyake *et al.*, 1986) and other plastics (Klotz *et al.*, 1985). First it should be noted that *C. albicans* adheres nonspecifically to surfaces. Hyphal form *C. albicans* was generally more adherent than yeast cells, and was hydrophobic

(Hazen and Hazen, 1988). However, a statistically significant correlation between the expression of cell surface hydrophobicity and adhesion to HeLa cells was not found. Hydrophobicity was greatest for stationary-phase yeast cells grown at 23°C, as opposed to 37°C, but only about 85% of the *C. albicans* strains tested fell into that category (Hazen and Hazen, 1988).

1.4.7. Thigmotrophism

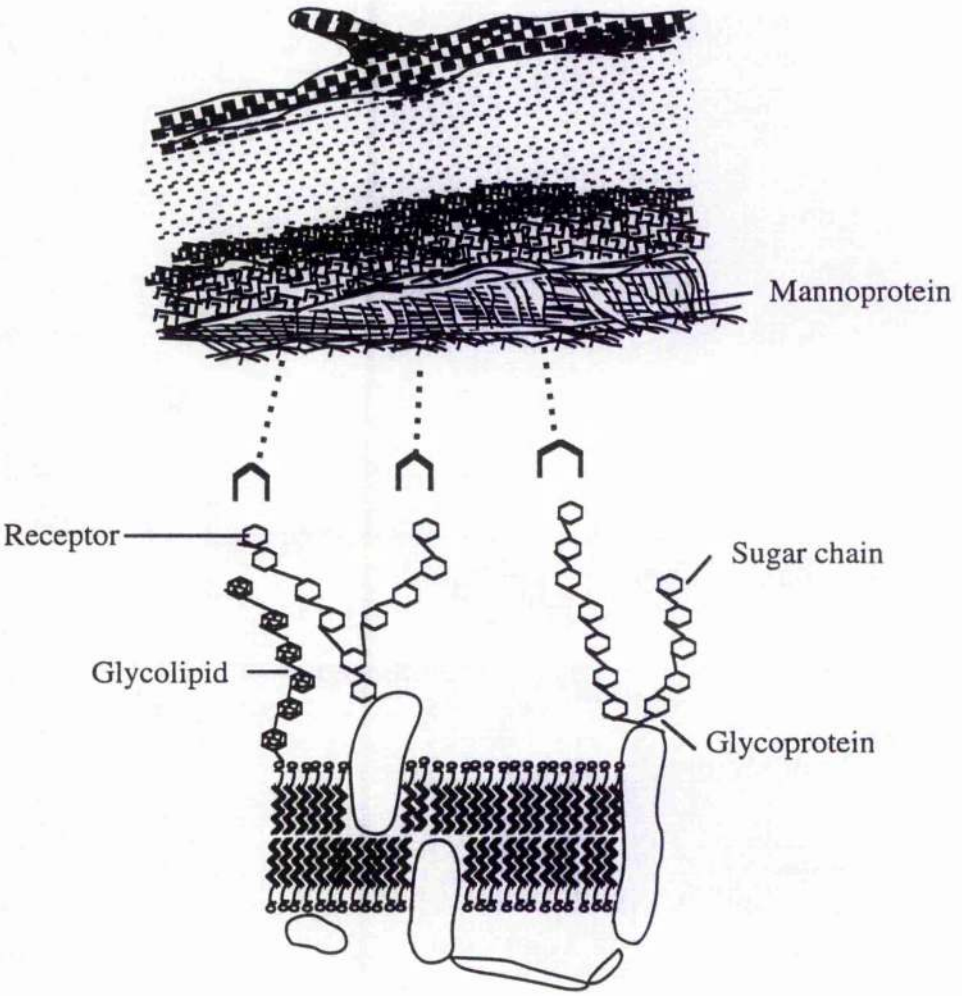
Thimotrophism (contact sensing) contributes significantly to virulence in some plant pathogenic fungi. When hyphal form *C. albicans* growing on the surface of Nucleopore membranes were placed on agar medium, there was a horizontal hyphal growth pattern, after which the organisms entered the pores and grew on the underside of the filters (Sherwood *et al.,* 1992). These directional changes probably required upregulation of genes, or the transcription of new genes. The directed growth may be important during the growth of *C. albicans* over mucosal epithelial surfaces.

1.5. ADHESION OF *CANDIDA* YEAST CELLS AND GERM TUBES TO SURFACES

1.5.1. Importance of Adhesion

C. albicans is capable of causing a range of superficial and systemic infections, and disseminated candidosis occurs in immunocompromised hosts, diabetics, the neonates, and postoperative patients. Adhesion of *C. albicans* to host tissues and plastic surfaces is the initial step in candidosis. Glycoproteins and glycolipids are ubiquitous macromolecules found on cell surfaces and presenting good sources of anchor sites or receptors for microorganisms to attach to host cells for colonization and subsequent infection (Figure 4). The adherence of *C. albicans* to host cell surface receptors is a complex process and may involve more than one mechanism of adherence and/or more than a single adhesin. Its involvement in the colonization processes of *C. albicans* came from work by Liljemark and Gibbons (1973) and King

Figure 4. Diagram illustrating interaction of *Candida* (above) with mammalian epithelial cell membrane (below), based on Ghannoum and Abu-Elteen (1991). The outer layer of the yeast cell wall is a mass of mannoprotein fibrils attaching to the glycoproteins extending from the epithelial membrane



et al. (1980). Colonization can occur at epithelial surfaces, including the mouth, the perineum, and vagina. When epithelial barriers are breached by implanted catheters or as a result of burns or surgical procedures, colonizing candidal species can invade the host.

Adhesion of *C. albicans* and other species to epithelium of gastrointestinal or genitourinary tract therefore stands as a critical first step in the pathogenesis of candidal infection. Having colonized the host's mucosal surfaces, *Candida* species may then invade beneath the mucosal barrier into the vascular space, where continued replication causes hematogenously disseminated disease (Figure 5). Invasion through the endothelial barrier may then give rise to metastases at distant sites including brain, retina, endocardium, liver, spleen, kidney, and bone (Odds, 1994).

1.5.2. Mechanisms of Adhesion of *C. albicans*

Candida adheres to epithelial surfaces either through direct interaction or indirectly through bridging, involving intermediate cells or components (Table 3).

1.5.2.1. Direct interactions

Adhesion of *Candida* to epithelial cells by direct interactions seems to occur in two phases, as nonspecific interactions (reversible or loose) and then specific (nonreversible, tight, intimate) interactions. The reversible phase lasts for about 20 min, whereafter the cell binding becomes irreversible (Sandin *et al.*, 1987).

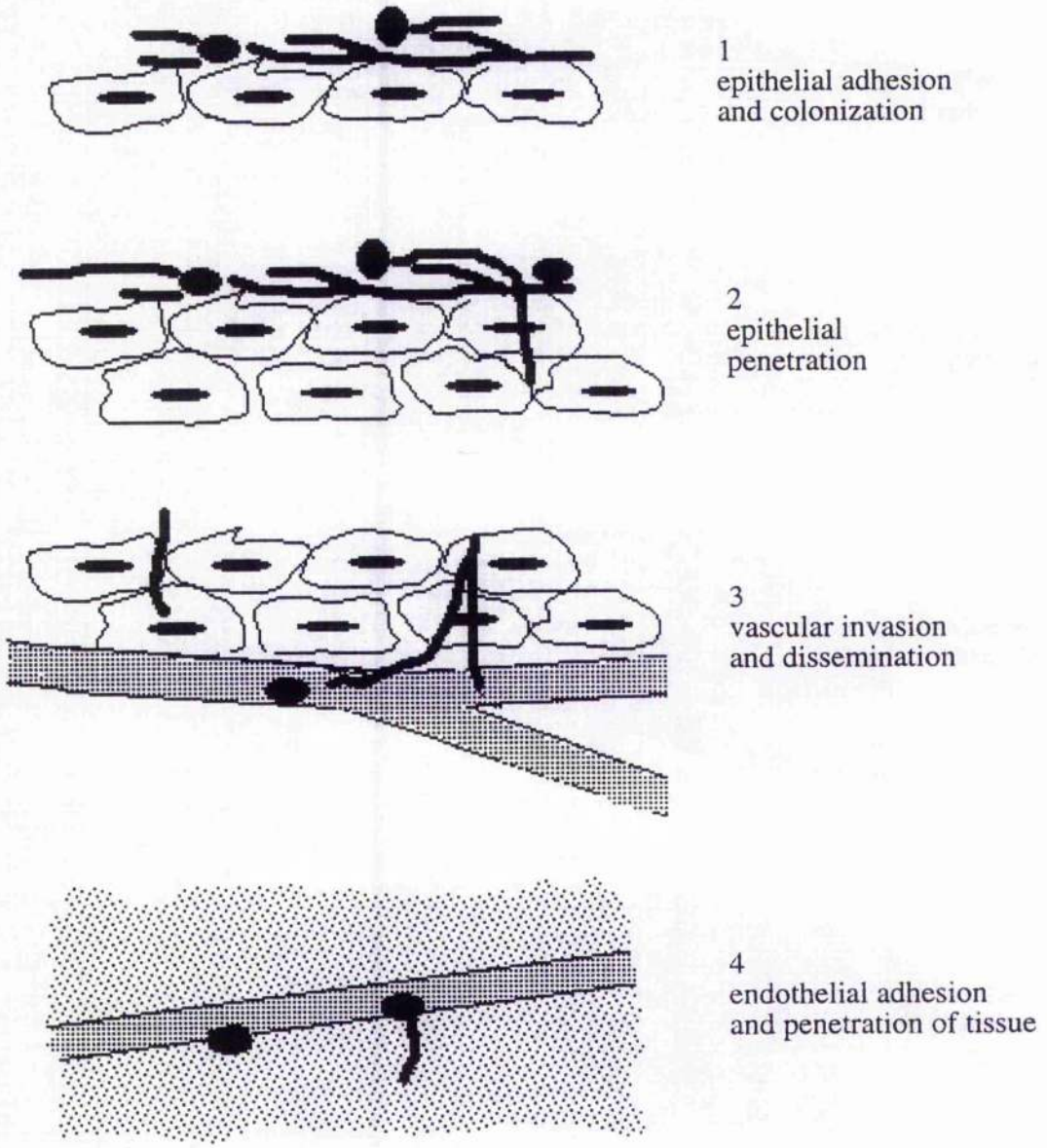
Nonspecific interactions are those associations between *Candida* and a cell surface that do not require a precise stereochemical fit. Whereas the specific interactions between *Candida* and with cell surfaces that require rigid stereochemical constraints and are referred to as irreversible, tight or intimate (Kennedy *et al.* 1992).

Candida and the cell surfaces to which it adheres both carry a net negative charge. Two negative charged surface cells theoretically should repulse each other. A possible physicochemical explanation for net adhesive interactions between two negatively charged surfaces is described as DLVO theory (long-range attraction) by

Table 3. Mechanisms of *C. albicans* adhesion (based on Ghannoum and Abu-Elteen, 1991)

Mechanism	Nature of mechanisms			References
	Active or passive	Specific or nonspecific	Direct or indirect	
Nonspecific Interactions	A P	N	D, I	Kennedy *et al.*, 1987; Klotz *et al.*, 1985; Mehentee and Hay, 1984; Minagi *et al.*, 1985.
Specific interactions (adhesin-receptor)	A	S	D, I	Calderone and Scheld, 1987; Collins-Lech *et al.*, 1984 ; Kimura and Pearsal 1980; Sandin *et al.*, 1982; Segal *et al.*, 1982a, b.
Germ tube penetration	A	S, N	D, I	Howlet and Squier, 1980; Marrie and Casterton 1981; Sobel *et al.*, 1984.
Enzyme production	A	S, N	D	Barrett-Bee *et al.*, 1985; Ghannoum and Abu Elteen 1986b; MacDonald and Odds, 1983; Ruchel *et al.*, 1992.
Coaggregation and coadhesion organisms	A	S, N	I	Centeno *et al.*, 1983; Kennedy *et al.*, 1987; Kennedy and Sandin, 1988.
Entraptment in tissue	P	N	D	Kennedy *et al.*, 1987; Pope and Cole, 1981.

Figure 5. Stages of a *Candida* infection (based on Odds, 1994)



Derjaguin and Landau (1941) and Verwey and Overbeek (1948) which was cited by Rotrosen *et al.* (1986) and Kennedy *et al.* (1992). This theory considers the energy of interaction of two charged particles of like sign and magnitude as the sum of the electrostatic energy of repulsion and the energy of attraction provided by London-van der Waal's forces. Repulsion and attractive forces vary indepedently with the distance between two cells. There is a net force attraction over relatively large distance (>10nm), and the cells are held in a state of weak, reversible association. The concentration of ion clouds on apposing surfaces at intermediate distance (1-10nm), causes a net repulsive force. Finally, if the intermediate zone of repulsion can be bridged, at the next step there is a strong net attractive force at a distance of 1nm and the cells can create irreversible interactions. This model is inadequate, however to describe close-range interactions, which are regulated by adhesion-receptor binding. *C. albicans* does not process sufficient kinetic energy to overcome the repulsion barrier, hence the relatively irreversible adhesion noted for the organism. But a bridge at the gap between the yeast and host cells can be created by the surface fibrillar layer and microvilli, cilia or by complex layer of glycocalyx or mucus respectively. Both type of interactions (reversible or irreversible binding) nevertheless are probably requisite for the adhesion to host mucosal surfaces or implanted biomaterials.

1.5.2.2. Indirect interactions

C. albicans may adhere to mucosal surfaces from the oral, gastrointestinal and urogenital systems by first binding to intermediary commensal microorganisms and/or binding ligands derived from the host (Ghannoum and Abu-Elteen, 1991). Coadhesion to adhered fungi, or bacteria and entrapment in the mucous gel, overlying the epithelium may all occur. The other mechanisms utilize host-derived binding ligands such as fibronectin (Calderone and Scheld, 1987), fibronogen (Bouali *et al.*, 1986), vitronectin and laminin (Bouchara *et al.*, 1990; Jakap *et al.*, 1993). Page

and Odds (1988) suggested that adhesion via plasma proteins such as albumin, transferrin and fibrinogen is more likely to be a secondary than a primary mechanism.

1.5.3. Factors Affecting Adhesion

1.5.3.1. Role of yeast factors

1.5.3.1.1. ***Species and strain differences*** *C. albicans* is undoubtly the most virulent *Candida* species, and it adheres to buccal and vaginal epithelial cells (ECs) to a significantly greater degree than other *Candida* species tested (King *et al.,* 1980). *C. tropicalis* and *C. stelloida* showed significant adherence to mucosal cells, while *C. parasilosis* adhered only to a slight degree. *C. guilliermondii, C. krusei* and *C. kefyr* failed to interact with ECs. Similar species differences have been reported for adhesion to epidermal corneocytes (Ray *et al.*, 1984), vascular endothelium (Klotz *et al.*, 1983), fibrin-platelet matrices (Maisch and Calderone, 1980) and intravenous catheters (Rotrosen *et al.*, 1983).

The differences in adherence between strain of *C. albicans,* which differed in virulence for mice, did not consistently show that the adherence capacities of the virulent strains were greater than the commensal strains (Kearns *et al.*, 1983). The adhesion and virulence of strains isolated from active infections were compared with those of two obtained from asymptomatic carriers. Growth in media containing a high concentration (500 mM) of sucrose or galactose enhanced the adherence of the strains obtained from active infections. By contrast, the asymptomatic strains in the same concentration of galactose showed only little or no increase in either property (McCourtie and Douglas, 1984). Less pathogenic *Candida* species as well as the non-pathogenic members of the genus also lack the cell surface versatility of the infective *C. albicans* strains (Critchley and Douglas, 1985). Other investigators have indicated that isolates of *C. albicans* from patients with vaginitis were significantly more adherent than isolates from asymptomatic carriers (Segal *et al.,* 1984). A correlation between adhesion, phospholipase activation and virulence for different isolates of *C. albicans* has also been reported by Barrett-Bee *et al.* (1985). In addition, Ghannoum

and Abu-Elteen (1986) showed a correlation between proteinase production, adherence and pathogenicity among *C. albicans* isolates of the same strain and of different strain types .

1.5.3.1.2. ***Adhesion-deficient mutants*** The importance of adhesion of *C. albicans* in the pathogenic process has been observed with mutants showing a reduced ability to adhere. Lehrer *et al.* (1986) found a spontaneous cerulenin-resistant mutant of *C. albicans* which adhered less readily *in vitro* to human vaginal epithelial cells than the parent strain and was less virulent in a mouse model of vaginal candidosis. Calderone *et al.* (1985) also showed that the same mutant failed to adhere to fibrin-platelet clots *in vitro*. The mutant readily bound C3d-coated erythrocytes, but rosetting with iC3b-coated erythrocytes was reduced by more than 50% (Ollert *et al.*, 1990).

1.5.3.1.3. ***Culture age and viability*** King *et al.* (1980) demonstrated that the growth phase of *C. albicans* had a marked influence on its adherence ability. They observed that stationary-phase yeast attached to vaginal epithelial cells in greater numbers than exponential-phase yeast, but Segal *et al.* (1982a) reported opposite results. Ghannoum and Abu-Elteen (1987) reported no significant difference in adherence ability of *C. albicans* at different growth phases.

Viable *C. albicans* cells adhered better to buccal epithelial cell (BEC) than heat- and formaldehyde-killed nonviable cells (Kimura and Pearsall, 1978; Maisch and Calderone, 1981). The enhancement of adhesion of *C. albicans* to HeLa cells and acrylic surfaces disappeared once yeast cells were heat-killed prior to incubation in sucrose-containing medium (Samaranayake and MacFarlane, 1981). However, heat-or formalin-killed yeasts have also been reported to show diminished adherence under conditions where germination did not occur (Samaranayake and MacFarlane, 1981; Sobel *et al.*, 1981).

1.5.3.1.4. ***Culture concentration*** There is apparently no detectable yeast attachment at concentrations below 10^4 yeast cells ml^{-1}. It has been showed that adhesion of *C. albicans* to BEC and VEC gradually increases when the ratio of yeast to ECs in the incubation mixtures is raised from 10:1 to 10,000:1 (Kimura and Pearsall, 1978; King *et al.*, 1980). According to Segal *et al.* (1982a), adherence of yeast to ECs was saturable on varying the concentration both yeast and vaginal epithelial cells. Klotz and Penn (1987) observed that high yeast inocula on a monolayer of human intestinal epithelium can cause greater aggregation of the yeasts rather than a random distribution of the yeast cells.

1.5.3.1.5. ***Germ-tube production*** Several investigators have suggested that the hyphal form is more virulent, by observing that active symptomatic infection by *C. albicans* is associated with the presence of hyphae whereas saprophytic *C. albicans* is almost invariably in the yeast form (Hill and Gerbhardt, 1956; Kozinn *et al.*, 1960). Young (1958) observed that the hyphal forms were the infective elements of the fungus and suggested that this observation may result from an inability of phagocytic cells to ingest hyphal cells but not yeast forms.

Kimura and Pearsall (1980) observed that germ tube production increased the adherence of *Candida* to epithelial cells. Sobel *et al.* (1984), emphasized the importance of hypha production in the pathogenesis of *Candida* vaginitis by experiments with a germ tube deficient mutant and its wild type. Martin *et al.* (1984) concluded that the pathogenic potential of *C. albicans* in the mouth depends on the formation of germ tubes. Tips of germ tubes of *C. albicans* can usually be seen as the entities that penetrate the host surface (Calderone *et al.*, 1984; Farrell *et al.*, 1983; Rotrosen *et al.*, 1985). However some authors have noticed yeast cells without germ tubes apparently burrowing into, or being engulfed by, host cells as a first stage of penetration (Calderone *et al.*, 1984; Klotz *et al.*, 1983; Pope and Cole, 1981). Cytochemical methods have been used to demonstrate that germ tube formation involves a significant reorganization of surface mannoprotein (Tronchin *et al.*, 1989).

1.5.3.2. Enviromental factors

1.5.3.2.1. ***Culture medium and temperature of growth*** Medium composition and temperature of growth are known to affect cell morphology of dimorphic fungi including *C. albicans* (Odds, 1988). Optimal adhesive activity was observed when the cells were grown in defined media and depended on the carbohydrate used (Kimura and Pearsall, 1980). Also, significant differences in adhesion to BEC were noted when *C. albicans* was grown in the same complex medium from different manufacturers and in different batches of medium from the same manufacturer (Kimura and Pearsall, 1980). Yeast incubated in media containing sucrose and glucose as a carbon source adhered better to acrylic strips than control yeast grown in sugar-free media . Growth in the presence of lactose and xylitol showed no significant difference when compared with control yeasts (Samaranayake *et al.*, 1980). Preincubation of *C. albicans* in the presence of a range of sucrose concentrations (50 - 500 mM) gave a significant correlation between the number of adherent yeasts to acrylic surfaces or ECs and the sucrose concentration (Samaranayake and MacFarlane, 1981; 1982). Similar results were also reported by others (Kearns *et al.*, 1983; McCourtie and Douglas 1981). The mechanism by which carbohydrates enhance adherence appears to be by the production of additional fibrillar-floccular layer on the yeast cell surface (Douglas, 1987a).

The growth temperature of *C. albicans* has also been shown to play a central role in its adhesion to various surfaces. Yeast harvested from cultures grown at 25 - 28°C adhered to VEC or BEC in much greater amount than those isolated from cultures grown at 37°C (Kennedy, and Sandin, 1988).

1.5.3.2.2. ***Hydrogen-ion concentration and carbon dioxide*** Optimal pH for adherence of yeast was reported to be in the range of 6 - 8 (Samaranayake and MacFarlane, 1982). This optimum also was cell-type specific: thus for the binding between cells of *C. albicans* and stomach mucosal cells, the pH ranged from 1.2 -

3.4, while optimal adherence to jejunal mucosal surfaces occured at neutral pH (Mehentee and Hay, 1989). Greater adhesion ability was observed when the yeast cells were incubated with VEC at pH 5 in PBS in ambient air supplemented with 10% CO_2 (Persi *et al.*, 1985).

1.5.3.2.3. ***Antibodies and saliva*** Secretory immunoglobulin A (s-IgA) against *Candida* inhibited adherence to buccal epithelial cells *in vitro*. An inverse correlation existed between the titre of salivary s-IgA anti-*Candida* antibodies and the degree of adhesion (Epstein *et al.*, 1982). The inhibitory effect was dependent upon the time of incubation of yeast cells with s-IgA and its concentration. Vudhichamnong *et al.*, (1982) similarly found that adhesion of *C. albicans* to buccal cells was inhibited by s-IgA isolated from breast milk.

Adherence of *C. albicans* to acrylic strips was inhibited when saliva-treated strips were used in assays or when the yeasts were suspended in saliva (McCourtie and Douglas, 1981). Adhesion of *C. albicans* to HeLa cells increased significantly under the influence of a salivary pellicle (Samaranayake and MacFarlane, 1982). Viable *C. albicans* cells, preincubated in saliva for 90 min at 37°C before being washed and mixed with ECs in PBS, adhered better than nonviable yeasts or yeasts preincubated in PBS (Kimura and Pearsall, 1978).

1.5.4. Role of Host Surfaces

1.5.4.1. Variation due to cell type and donor

In vitro adherence of *C. albicans* may be influenced by the mucosal cell donor, the time of collection of mucosal cells, and the body site of their origin. *In vitro* assays of adherence of *C. albicans* to human epithelial cells from 24 donors showed statistically significant differences in the number of attached yeast cells between individuals (Sandin *et al.*, 1987). Sex of donor did not have any significant influence on adhesion. Yeast attachment to mucosal cells varied significantly within subjects in

relation to time over 5 days. Cells from some donors showed greater date-to-date variation in yeast adhesion than others.

Althought Sobel *et al.* (1982) showed that there was no significant difference in adherence of *C. albicans* to BECs and VECs, Calderone *et al.*, (1984) and Collins-Lech *et al.* (1984) found that *C. albicans* adhered better to buccal cells than to corneocytes. Yeast adherence was highest to BECs and lowest to urinary tract cells, whereas VECs were intermediate (Olsen, 1990).

1.5.4.2. Blood-group antigens

The ABO[H] and Lewis group antigens, which all possess L-fucose residues seem to be responsible for *C. albicans* adhesion to epithelial cells. Correlations between ABO[H] blood group and susceptibility to infection have been reported (Deresinski, *et al.*, 1979) and blood group determinants like L-fucose have been shown to act as microorganism receptors (Jones and Freter, 1976). ABO[H] antigens occur on the red cell membrane, on platelets and on tissues and are present on the surface of many cells including mucosal epithelium (Mollicone *et al.*, 1985). All ABO[H] antigens are synthesized on a type 2 chain, up to 80 % of these chains being bound to protein, and some 20 % being glycoproteins. In secretions, ABO[H] structures are found as glycoproteins on both chain types (Rege *et al.*, 1963). The investigators group also reported that non-secretion of ABO[H] blood group antigens was associated with susceptibility to superficial fungus infections (Blackwell *et al.*, 1990). In healthy subjects, blood group O and non-secretion group antigens were separate, and cumulative risk factors for oral carriage of *C. albicans* (Burford-Mason *et al.*, 1988). But, Blackwell *et al.* (1990) showed that there was no association between ABO[H] blood group and carriage.

Tosh and Douglas (1992) purified the fucose-binding protein as an adhesin of *C. albicans* and obtained it by affinity adsorption with the trisaccharide determinant of the H (type 2) blood group antigens. The purified adhesin was devoid of carbohydrate, and gave 80 % inhibition of yeast adhesion to buccal epithelial cells. In

the study of Brassart *et al.* (1991), various glycopeptides and structurally defined oligosaccharides inhibited *Candida* adhesion by up to 55%, but inhibition was completely abolished if fucose residues were removed from the compounds by mild acid hydrolosis. When oligosaccharides were used as inhibitors, the minimal structural requirement for activity was the Fuc α1->2 Gal β determinant of the H-antigen which is found on all the blood-group substances of the ABO system (Brassart *et al.*, 1991).

The Lewis system which exists in two forms Le^a and Le^b, differs from the other antigens in not being found on red cells but being present in plasma and in body secretions such as saliva. May *et al.* (1989) suggested that the $Lewis^a$ antigen may be one of the receptors for some yeast strains. This was tested by examining the binding of *C. albicans* to epithelial cells from a non-secretor. Such binding was inhibited by pretreating with anti $Lewis^a$-antigen, while none of the other retreatments produced statistically significant differences (May *et al.*, 1989; Tosh and Douglas, 1991). Moreover the Lewis blood-group antigens which are also fucosylated, were totally inactive (Brassart *et al.*, 1991). These results show that the ability of *C. albicans* to bind the Lewis ^{a} antigen is strain-dependent (May *et al.*, 1989).

1.5.5. Adhesion to Host Surfaces

1.5.5.1. Epithelial cells

C. albicans adhesion to epithelial surface is important since infections of the mouth and vagina are the most common forms of superficial candidosis. For this reason, human buccal cells (BEC), and vaginal epithelial cells (VEC) have been most frequently used (Table 4). Liljemak and Gibbons (1973) were the first to measure the ability of *C. albicans* to adhere to host cells. Kimura and Pearsall (1978) used polycarbonate filters with a pore size that allowed passage of unattached yeast but not of epithelial cells. Adherent yeast cells on epithelial cells were fixed, stained and counted directly on the transparent polycarbonate filter. Douglas *et al.* (1981) modified this method and used it to observe adhesion of yeast to BEC or VEC. This

Table 4. Adhesion of *C. albicans* to host cells

Host cells	References
Buccal epithelial	Ghannoum *et al.*, 1986; Ghannoum *et al.*, 1987; Kearns *et al.*, 1983; Kennedy and Sandin 1988; Critchley and Douglas 1985; Kimura and Pearsall, 1978; Sandin *et al.*, 1982; Sobel *et al.*, 1981.
Vaginal epithelial	Segal *et al.*, 1982a; Sobel *et al.*, 1982.
Uroepithelial	Centeno *et al.* 1983.
Epidermal cornecytes	Collins-Lech *et al.*, 1984; Ray *et al.*, 1984.
Gastrointestinal epithelial	Pope and Cole *et al.*, 1981; Segal and Sevage 1986.
Endothelial	Klotz *et al.*, 1983, Klotz 1987; Klotz and Penn 1987; Klotz and Maca 1988
Cervical epithelial	Farrell *et al.*, 1983
Intestinal cell lines	Mehentee and Hay, 1989.
HeLa	Samarayanaka and MacFarlane, 1981, 1982
Fibrin-platelet	Maish and Calderone, 1980, 1981; Calderone *et al.*, 1985.

method has also been used to with the hyphal form cells (Sandin *et al.*, 1982). It allows measurement of individual epithelial cells. King *et al.* (1980) used radioactively-labelled yeast to measure adherence of several *Candida* species to BEC and VEC.

Generally, yeast form *C. albicans* has been used in adhesion assays. However there is general agreement that the hyphal-form cells adhere to host surfaces better than the yeast forms *in vivo* (Taschdjian and Kozinn., 1957). In the early literature, germ tube formation promotes adhesion of *C. albicans* to epithelial cells (Kimura and Pearsall, 1980; Sandin *et al.*, 1982; Sobel *et al.*, 1984). Generally, tips of hyphal form cells can usually be seen as the entities that penetrate the epithelial cells (Howlet and Squire, 1980; Calderone *et al.*, 1984, Sobel *et al.*, 1984).

Mechanisms of adherence of *C. albicans* to epithelial cells can be categorized as involving host cell protein (protein-protein) lectin-like (protein-carbohydrate) and incomplete defined interactions with chitin, lipid or mannan of the cells wall (Table 2).

1.5.5.2. Endothelial cells

Epithelial cell adhesion by *C. albicans* is due to first contact of the fungus with the host, whereas with endothelial cells, adhesion to the cells occurs as a late state after invasion by the fungus or contamination via an intracellular catheter (Klotz *et al.*, 1983). *C. albicans* was more avidly bound to porcine vascular endothelium *in vitro* than other *Candida* spp. such as *C. tropicalis*, *C. krusei* and *C. parapsilosis* (Klotz *et al.*, 1983). A crude extract from heat-exposed *C. albicans* inhibited adherence of the fungus to human umbilical vein endothelial cells. In the same experiment purified mannan, mannose, glucose, lactose, galactose, *N*-acetylglucosamine and sucrose did not inhibit *Candida* adherence to the host cells *in vitro*. Although the hyphal-form cells were observed to slightly augment adherence, the yeast-form cells were able to attach to epithelium (Rotrosen *et al.*, 1985). The binding of yeast to human intestinal epithelium was blocked by mucin and monosaccharides such as glucose and galactose

but not by mannose and fucose (Klotz and Penn, 1987). The latter concluded that *C. albicans* appears to adhere to human intestinal epithelium by several mechanisms such as adhesin-receptor interactions, nonspecific hydrophobic and ionic bonding and aggregation and coadherence. Mayer *et al.* (1992) observed that hyphal form *C. albicans* was attached to human umbilical vein endothelial cells *in vitro.*

Hostetter, (1994a, b) recently reviewed mechanisms of adherence of *C. albicans* to endothelial cells and described interactions between protein on the cell surface of the fungus and proteins of host cells. Also *C. albicans* possesses substances that bind complement, fibrinogen, fibronectin, laminin, vitronectin. Detailed information about host proteins involved in binding was given in 1.4.1.2.

1.5.5.3. Extracellular matrix

The pathogenesis of disseminated candidosis is not well understood, but adherence of *C. albicans* to endothelial cells or subendothelial extracellular matrix (ECM) may be involved in blood-borne disease (Klotz, 1987; Klotz and Maca, 1988). These authors observed that *C. albicans* yeast cells adhered in greater numbers and with greater avidity to exposed subendothelial ECM than to confluent endothelium. The adherence of the yeast cells could be abolished by exogenous plasma fibronectin (Klotz and Smith, 1991) and by a synthetic arginine-glycine-aspartic acid (RGD) peptide (Klotz and Smith, 1990) and RGD-containing peptide (Klotz *et al.*, 1992). Sawyer *et al.* (1992a) showed that RGD peptides bind to a receptor on the surface of *C. albicans*, thereby increasing hepatic, and presumably Kupffer cell, killing of the fungus. They suggested that natural or synthetic RGD peptides may serve as opsonin promoting *C. albicans* killing by Kupffer cells.

Recently, Klotz *et al.* (1993) isolated cell wall protein from columns of fibronectin or gelatin coupled to agarose. In the experiment, yeast extracts from *C. albicans* were applied to the column. The fungal protein had a relative molecular mass of 60 kDa and eluted with 10mM EDTA, 2% α-methylmannopyranoside or an RGD-containing peptide. Immunoblot analysis of the yeast extracts demonstrated that

anti-integrin antibodies to the human fibronection, vitronectin and complement receptor cross-reacted with the 60 kDa glycoprotein. The adherence of *C. albicans* to fibronectin as an extracellular matrix (ECM) protein is believed to be important in the pathogenesis of such specific entities as disseminated candidosis (Klotz and Smith 1991) and candidal endocarditis (Calderone and Scheld, 1987).

1.5.5.4. Fibrin-platelet matrices

Platelets are deposited at sites of vascular injury surrounding intravenous catheters and vascular prostheses. These platelet target sites are also favoured sites for endovascular infections with *Candida* in the yeast form. Infective endocarditis is characterized by colonization of heart valves by *C. albicans*. Maisch and Calderone (1981) observed that *C. albicans* was highly adherent and they suggested that surface mannan played a role in the adhesion mechanism. Platelet extract which was derivated from the platelet-rich plasma supported germ tube formation from yeast cells (Skerl *et al.*, 1981). These results suggested that aggregated platelets *in vivo* promote germ tube formation by *C. albicans* and can cause disease. The other study demostrated that *in vivo* colonization of rabbit endocardium by *C. albicans* was reduced by antibodies to the fungus (Scheld *et al.*, 1983).

Two cerulenin-resistant mutants failed to adhere to fibrin-platelet matrices and were relatively avirulent in a rabbit endocarditis model (Calderone *et al.*, 1985). Exposed subendothelial extracellular matrix induced the aggregation of platelets and promoted avid binding by the yeast (Klotz *et al.*, 1989). Experimentally infected mice showed a rapid and significant fixation of platelets on *C. albicans* in the blood stream (Mahaza *et al.*, 1991). Hyphal form cells were more rapidly attached to platelets than the yeast form.

Calderone *et al.* (1994) recently developed an *in vitro* method to explore the *in vivo* mechanism. Hyphal forms of *C. albicans* were immobilized on a Sepharose column, the platelet suspension was then poured in and the *Candida*-adhered platelets counted. The percentage of adherent platelets varied linearly with the concentration of

the hyphal forms in the column. Preincubation of the hyphal-form cells with platelet extracts prior to their incorporation into the column resulted in greatly reduced platelet adhesion to the germ tubes. Anti-hyphal form or anti-yeast form antibodies also inhibited the binding of platelets to hyphal form cells and in a dose-dependent manner. But anti-mannan antibodies did not show any significant effect. Neither carbohydrates nor peptides with RGDS motifs inhibited the binding of hyphal-form cells to platelets. The relationship between *C. albicans* and platelets may allow better understanding of the invasion mechanism of the fungus.

1.5.5.5. Effect of commensal bacteria

Candida -mucosal interactions may be antagonized by bacteria and thereby reduce colonization and invasion by this fungus. Members of the Enterobacteriaceae such as *Salmonella* spp. and *Shigella flexneri* (Barber *et al.*, 1973), *E. coli* and *Proteus* spp. (Cormane and Goslings, 1963) and anaerobic bacteria (Kennedy, 1981) have all been found to supress *C. albicans* growth. Centeno *et al.* (1983) reported enhancement of yeast attachment by *E. coli* and *K. pneumoniae* by mannose-sensetive pili on the bacteria. Liljemark and Gibbons (1973) found that twice as many yeasts attached to germ-free rat cells than to cells from conventional animals, suggesting that mixed strains of *Streptococcus salivarius* and *Streptococcus miteor* and mixed salivary bacteria may inhibit *Candida* colonization of mucosal surfaces. Preincubating these bacteria with HeLa cells reduced attachment of the yeast cells (Samaranayake and MacFarlane, 1982). Sobel *et al.* (1981) found that preincubation of VEC with two vaginal isolates of *Lactobacillus* spp. also significantly decreased yeast adhesion.

1.5.6. Adhesion to Inert Surfaces

C. albicans has the ability to adhere to inert surfaces such as dentures (Samaranayake and MacFarlane, 1980), contact lenses (Butrus and Klotz, 1986), catheters (Rotrosen, *et al.*, 1983), and prostheses such as heart valves. Invasion of the human host by the

fungus can therefore occur by adherence to such plastics followed by its dissemination through the vascular system (Rotrosen *et al.*, 1986). *C. albicans* through its ability to bind to dentures may cause denture stomatitis (Samaranayake and MacFarlane, 1980, Samaranayake *et al.*, 1980; McCourtie and Douglas, 1981, 1984). Samaranayake and MacFarlane (1980) incubated small, transparent acrylic strips with the yeast cells and after removing the unattached organisms by rinsing, the cells were counted under a microscope. Similar methods have been applied to measure yeast adhesion to a variety of denture resin materials (Minagi *et al.*, 1985) and plastics (Klotz *et al.*, 1985).

The adhesion of *C. albicans* to plastic depends on growth conditions. For example, adherence of *C. albicans* grown in 500 mM galactose on acrylic strips was better than those grown in 50 mM glucose (Samaranayake and MacFarlane, 1980; McCourtie and Douglas 1981). Mannoprotein which was isolated from cell walls of *C. albicans* grown in a high concentration of galactose reduced adherence to acrylic as it did to buccal epithelial cells (McCourtie and Douglas, 1985). Hydrophobic interactions appeared to be of primary importance in the adhesion of *Candida* species to plastic surface *in vitro* (Minagi *et al.*, 1985; Klotz *et al.*, 1985; Critchley and Douglas, 1985). Adherence of *C. albicans* to the acrylic was prevented by chitin derivative in two different systems: chitin derivatives present during the adherence assay and acrylic pieces pretreated with chitin derivates prior to the assay (Segal *et al.*, 1992).

Tronchin *et al.* (1988) observed that hyphal-form cells were more adherent than yeast forms. To produce germ tubes, the yeast-form cells in stationary phase in Modified Lee Medium without aminoacids were inoculated into 199 medium at pH 6.7 and incubated at 37°C in polystyrene petri dishes for 3 h. After removing the hyphal-form cells, the petri dishes were rinsed with distilled water. Con A-coated latex beads were then used to detect germ tube mannoproteins on the petri dish. Con A-coated gold labelled hyphal-form cells were observed by electron microscopy, showing that the germ tube portions of the cells have fibrils which contained

mannoproteins. These fibrillar structure of the cell wall may be reponsible for attachment to the plastic surfaces. Dithiothreitol and iodoacetamide treatment of washed petri dishes allowed solublization of the fibrillar structure. The material was analysed by SDS-PAGE and revealed that four specific proteins, 60, 68, 200 and >200 kDa were involved in *C. albicans* germ-tube adherence to plastic. Using the same system to isolate hyphal-form cell wall extracts, two of these protein components (60, 68 kDa) interacted with host proteins such as laminin, fibrinogen or C3d (Tronchin *et al.*, 1989; Bouchara *et al.*, 1990). Adherence of yeast or hyphal-form cells which had been grown in YNBGal and 199 Medium, to whole saliva-coated acrylic was not different (Vasilas *et al.*, 1992). Recently, Hawser and Douglas (1994) showed that growth of *C. albicans* as biofilms on plastic catheters in medium containing 500 mM galactose or 50 mM glucose reached a maximum after 48 h and then declined. However, the cell yield was lower with the cells grown in the glucose medium. The results also confirmed that *C. albicans* in 500 mM galactose medium synthesises a fibrillar mannoprotein which was responsible for the increased yeast adhesion to inert surfaces such as acrylic plastic *in vitro* (McCourtie and Douglas, 1981; Hawser and Douglas, 1994). According to Vasilas *et al.* (1992), yeast and hyphal-forms of a wild type strain did not differ substantially in their ability to adhere to saliva-coated acrylic *in vitro* but the germ tube-deficient mutant had lowest binding capacity. Hyphal and yeast form cells were also observed as biofilms on the plastic catheters, but the yeast form only was observed on an agar surface of the same medium (or in liquid culture). These events indicate that a contact-induced regulation of gene expression at the molecular level may be occurring during morphogenesis (Hawser and Douglas, 1994).

These results demonstrated that the protein portion of the mannoprotein layer influences adhesion of *C. albicans*. Catheters and the other prosthetic devices *in vivo* are rapidly coated with host proteins such as albumin, laminin, fibrin, fibrinogen, fibronectin, and vitronectin. Some of these proteins could be candidal adhesion and biofilm promoters on the catheters.

2. OBJECT OF RESEARCH

1. The primary object of this research was to determine whether germ tubes of *Candida albicans* had a) lectin-like adhesins, and b) fibronectin adhesins, additional to those known for fibrinogen and laminin.

2. Primarily to gain experience with *Candida* adhesion processes, a study was made of the possible inhibitory effect of germ tube surface mannoprotein on adhesion of yeast cells of *C. albicans* to buccal epithelial cells.

3. Further questions of particular interest were a) whether lectin-like adhesins of hyphal form *C. albicans* would be concentrated at the growth tip, and b) whether the germ tube portion of hyphal form *C. albicans* would possess fibronectin adhesins in greater abundance than on the yeast cell portion of the hyphal-form cells. For these questions, the application of fluorescence-labelled neoglycoproteins was explored.

4. Finally, the research was focused on investigating, with fibronectin-coated colloidal gold, the pattern of distribution of fibronectin adhesins on the yeast and hyphal form cells.

3. MATERIALS AND METHODS

3.1. *CANDIDA ALBICANS* STRAINS

Three *C. albicans* strains were used in this study. Strain GDH 2346 had been isolated at Glasgow Dental Hospital from a patient with denture stomatitis and was supplied to Dr. L. J. Douglas (IBLS, Division of Infection and Immunity, University of Glasgow) by Dr. L. P. Samaranayake. It was deposited in the National Collection of Yeast Cultures, Food Research Institute, Norwich, England as strain NCYC 1467. The strain was described by Samaranayake and MacFarlane (1980).

The other organisms used in this study were strain MEN, originally obtained from an eye infection by Whelan and Magee (1981) and strain MM2002, a mutant which was unable to grow in the hyphal form and which had been isolated from the parental strain by a physical separation technique derived (Cannon, 1986). Both strains were obtained from Dr. R. D. Cannon, (Experimental Oral Biology Unit, Faculty of Dentistry, University of Otago, Dunedin, New Zealand) by Mr A. I. Tahir (IBLS, Division of Infection and Immunity, University of Glasgow). Previous studies revealed that apart from germ tube production in horse serum at 37°C, the wild strain and the mutant shared identical characteristics (Cannon, 1986).

All three strains were supplied as freeze-dried samples from which further freeze-dried ampoules were prepared.

3.2. MEDIA FOR *CANDIDA ALBICANS*

3.2.1. Sabouraud Dextrose Agar and Broth

Sabouraud Dextrose Agar (Difco) was dissolved in DW, usually in 500 ml amounts and sterilized at 121°C for 15 min according to manufacturer's instructions. Slopes (10 ml medium in universal containers) and plates (20 ml medium in 90 mm plastic petri dishes) were prepared.

Sabouraud Dextrose Broth (Difco) was prepared similarly and dispensed in 10 ml amounts in universal containers.

3.2.2. Yeast Nitrogen Base Media

Yeast Nitrogen Base Medium, YNB (Difco) was used for two different media both of which were normally prepared in batches of 1 L. YNBGal was YNB supplemented with 500 mM galactose (90 g L^{-1}) and was distributed in 50 ml in 250 ml Erlenmeyer flasks and sterilized in a pressure cooker at 110°C (10 lb sq in^{-1}) for 10 min. This medium had been used extensively by Douglas and coworkers (McCourtie and Douglas 1981, 1985; Critchley and Douglas 1987a,b). For those experiments where it was critical for the medium to be free from possible precipitates and where it had been stored, an additional filtration through a 0.45 µm membrane filter was done.

YNB was also supplemented with 1% (w/v) glucose and 0.15 % (w/v) L-asparagine as described by Mayer *et al.* (1991) and sterilized as YNBGal.

3.2.3. Modified Lee Medium without Amino Acids

Lee medium (Lee *et al.*, 1975) as modified by Tronchin, *et al.*, (1988) containing the following: $(NH_4)_2SO_4$, 5.0 g ; $MgSO_4.7H_2O$, 0.2 g ; K_2HPO_4, 2.5 g ; NaCl , 5.0 g; glucose, 10.0 g, dissolved to 1.0 L in DW and the pH was adjusted to pH 6.8 with HCl. It was sterilized in 500 ml aliquots in a pressure cooker at 110°C (10 lb sq in^{-1}) for 10 min. After cooling, biotin (0.04 g) from Sigma was dissolved in 20 ml of the sterilized medium and added by syringe and membrane filter (0.45 µm) as 10 ml per 500 ml.

3.2.4. 199 Medium

IX Medium 199 (Modified) with Hank's salts and 20 mM HEPES buffer, but without glutamine and sodium bicarbonate, was bought from Flow Laboratories, Irvine Scotland. Its pH was adjusted with HCl to 6.7 and filtered through a 0.45 µm membrane filter. The full formula is presented in Appendix 2.

3.2.5. Glucose-Glycine Medium

Glucose-Glycine Medium (GG) as described by Muerkoester *et al.* (1979) consisted of 1% (w/v) glucose, 1% (w/v) glycine and 0.1% (w/v) yeast extract (Difco). The medium was prepared by dissolving the glycine (10.0 g) and the yeast extract (1.0 g) in 500 ml DW and adjusted to pH 7.5 with solid $NaHCO_3$ and autoclaved. The glucose solution (10.0 g in 500 ml DW) was autoclaved separately and added to the glycine-yeast extract. The pH of the final medium was adjusted to 7.2 with HCl, followed by sterile filtration.

3.2.6. Sucrose-Gelatin Medium

Sucrose-Gelatin Medium (SG) as described by Mayer *et al.* (1992), consisted of 0.15% (w/v) sucrose (BDH) and 0.075% gelatin (w/v) (Oxoid). The ingredients were dissolved in DW, the pH adjusted to 7.4 and the medium sterilized in a pressure cooker at 110°C (10 lb sq in^{-1}) for 10 min.

3.3. GROWTH OF *CANDIDA ALBICANS*

3.3.1. Maintenance of Stock Cultures

For freeze-drying, the yeasts were grown on Sabouraud dextrose agar at 37°C for 24 h and the cells harvested in sterile 0.15 M phosphate-buffered saline (PBS) pH 7.2. The cells were pelleted by centrifugation (2332 g, 5 min, Megafuge 1.0, Heraeus, Sepatech), washed in PBS and resuspended in about 0.5 ml of 2% (w/v) sterilized skimmed milk. Sterile glass ampoules were each charged aseptically with single drops of the suspension and the ampoules frozen at -70°C for about 20 min. They were then dried in a centrifugal freeze-drier (Edward's High Vacuum Ltd., Sussex) and flame-sealed *in vacuum*. The ampoules were kept at -20°C. For resuscitation, an ampoule was opened and 2 drops of Sabouraud Dextrose Broth added. After 20 min, the suspension

of cells was inoculated onto a slope of Sabouraud Dextrose Agar, incubated at 37°C for 24 h and stored at 4°C. The slope was subcultured monthly and every second month, cultures were replaced by new ones freshly grown from the freeze dried stocks.

All buffers in these experiments were prepared according to Appendix 3.

3.3.2. Production of Yeast Cells

3.3.2.1. Cells for adhesion assays and labelling experiments

C. albicans was inoculated from stock cultures into YNBGal and incubated at 37°C for 18 h with orbital shaking at 150 rpm. This culture (5 ml) was used to inoculate a second flask of YNBGal medium (45 ml) and incubated at 37°C for 24 h on the orbital shaker. The cells in the stationary phase were harvested by centrifugation (2332 g, 5 min, Megafuge 1.0, Heraeus, Sepatech) and were used immediately after preparation.

3.3.2.2. Starved cells for germ tube production

Starved cells of *C. albicans* were prepared by the method of Tronchin *et al.* (1988). Stock cultures were inoculated into 250 ml flasks containing 50 ml Modified Lee Medium without Amino Acids (MLMwAA) and incubated at 25°C for 36 h with orbital shaking at 150 rpm. The cells were harvested by centrifugation (2332 g, 5 min, Megafuge 1.0, Heraeus, Sepatech) and washed twice with DW and resuspended in 2 ml DW. This suspension was added dropwise into 10 ml 199 Medium at either pH 6.7 or 4.0, until a concentration of 10^7 cells ml^{-1} had been reached, as determined by improved Neubauer haemocytometer. The pH 6.7 culture was then incubated at 22°C and the pH 4.0 culture, at 37°C for up to 5 h.

3.3.3. Production of Germ Tubes

During the course of this investigation three different procedures were used to produce germ tubes.

3.3.3.1. Method of Tronchin *et al.* (1988)

In the method finally adopted, germ tubes were produced by the method of Tronchin *et al.* (1988). Yeast cells were inoculated from stock cultures into 50 ml Modified Lee Medium (section 3.2.3) in a 250 ml conical flask. After incubation at 25°C for 36 h with constant shaking at 150 rpm, the starved cells so produced were harvested by centrifugation (2332 g, 5 min, Megafuge 1.0, Heraeus, Sepatech) and washed twice with DW. The final pellet of starved yeast cells was resuspended in 2 ml DW and added dropwise to 10 ml 199 Medium (section 3.2.4) in a 50 ml conical flask to give a count of 10^7 cells ml^{-1}. The culture was then incubated at 37 °C for up to 5 h with constant shaking at 150 rpm (Figure 6). Samples were removed at intervals and examined as wet preparations at 400x to monitor the appearance of germ tubes. Incubation was usually stopped at 2-2.5 h when the percentage conversion to germ tubes was about 90%.

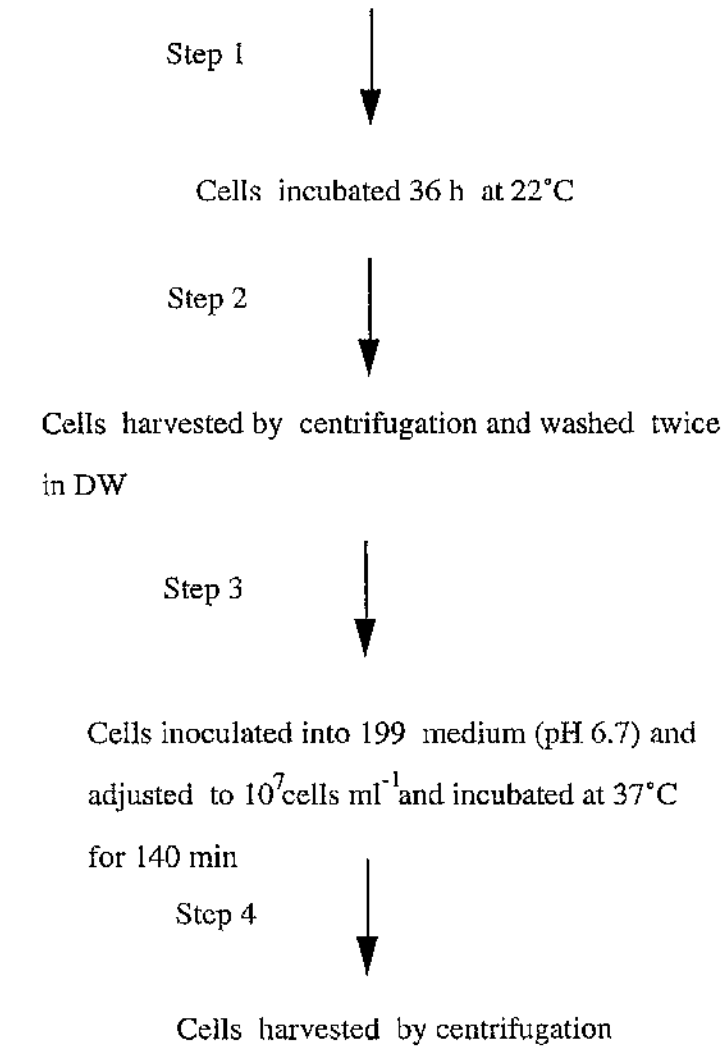
3.3.3.2. Method of Muerkoester *et al.* (1979)

An alternative method for preparing germ tubes was one of those described by Muerkoester *et al.* (1979). The procedure was basically the same as above except that the starved cells instead of being inoculated into 199 Medium were inoculated into Glucose-Glycine Medium with incubation and conversion to germ tubes as above 90.

3.3.3.3. Method of Mayer *et al.* (1992)

Unlike the two above procedures, a three-stage process was employed. Yeast cells were inoculated into 50 ml YNB containing 1% (w/v) dextrose and 0.15 % (w/v) L-asparagine in a 250 ml flask and incubated at 27°C overnight on a shaker at 150 rpm. From the overnight culture, 5 ml was inoculated into fresh 45 ml of the same medium and grown at 27°C for 24 h, again with shaking at 150 rpm. In the third stage, 10 ml from stage two was harvested, the cells washed twice in saline and resuspended in 2 ml saline. This suspension was added dropwise to 10 ml SG Medium (section 3.2.6) in a 50 ml conical flask to give a count of 10^7 cells ml^{-1}. The culture was then incubated at 37 °C with constant shaking at 150 rpm for up to 140 min.

Figure 6. Flow chart of standard protocol for producing germ tubes by *C. albicans*



3.4. ADHERENCE OF HYPHAL FORM *C. ALBICANS* TO PETRI DISHES

3.4.1. Germ Tube Formation on Petri Dishes

The procedure used was that described by Tronchin *et al.* (1988). Germ tubes were produced according to section 3.3.3.1 above, except that the cell concentration was increased to $2x10^7ml^{-1}$. This suspension in 15 ml aliquots in 199 Medium was poured into four petri dishes of diameter 90 mm and made from biologically neutral polystyrene (Philip Harris Scientific). Three of the petri dishes were incubated for 3 h at 37°C without shaking and the fourth at 22°C.

3.4.2. Germ Tube Mannoprotein Adhesins on Petri Dishes

3.4.2.1. Con A-coated latex beads

Concanavalin A (Con A)-coated latex beads were used to detect *C. albicans* adhesins on the petri dishes prepared as just described. The coating procedure was based on the methods of Quash *et al.* (1978) and Tronchin *et al.* (1987; 1988). Carboxylated polystyrene latex beads (0.9 µm, Sigma) were obtained as a 10% latex suspension in 0.14 M NaCl. The bead suspension (200 µl) in Eppendorf tubes was centrifuged with a microcentrifuge (1523 g for 10 min, Biofuge 13 Heraeus, Sepatech) and washed four times with 0.01 M borate HCl pH 8.1 (BBS). The washed beads, transferred to a universal container, were mixed with 2 ml of 1 mg ml^{-1} Con A (Sigma) and 1 mg 1-Ethyl-3-Dimethylamino propyl carbodiimide HCl, (EDC, Sigma) was added. The mixture was incubated for 2 h at 37°C with occasional shaking. After two washes with 20 ml BBS containing 1% (w/v) BSA (Sigma) (BBS-BSA), the latex beads coated with Con A were suspended in 20 ml BBS-BSA containing 0.1 % (v/v) Tween 20.

3.4.2.2. Detection of adhesins

The yeast and hyphal-form cells (section 3.4.1) were removed from the plastic surface of the petri dishes with a plastic scraper (Falcon, 3086-Becton Dickinson) designed for tissue culture work. The petri dishes were then washed three times with 10 ml DW and examined microscopically for completeness of removal of germ tubes. The dishes were then 'blocked' by incubation with 15 ml phosphate buffered saline (PBS) containing 0.5 % (w/v) BSA (PBS-BSA) for 1 h at room temperature, to prevent nonspecific binding.

The suspension of latex beads coated with Con A (5 ml) was poured into each petri dish and incubated 30 min at room temperature to allow binding. Excess latex beads were withdrawn with a pipette and the dishes washed three times with 10 ml PBS-BSA prior to microscopical observation of the latex beads on the petri dishes.

In each experiment, two control plates for the hyphal-form cells were prepared. One consisted of a scraped and blocked dish into which was poured a preincubated (30 min at room temperature) mixture of 0.2 M α-methyl-D-mannoside (Sigma) with Con A-coated latex beads. The other control was a dish which had not been treated with germ tubes but was subsequently incubated with Con A-coated latex beads.

3.5. PURIFICATION AND CHARACTERIZATION OF GERM TUBE ADHESINS

The hyphal-form cells were removed from the plastic surface with a scraper after incubation for 3 h. Petri dishes were washed three times with DW. Each washed petri dish was filled with 15 ml tris HCl-mannitol buffer at pH 7.5 and dithiothreitol (Sigma) (0.5 M) in tris HCl-mannitol buffer added to a final concentration of 12 mM . After incubation for 2 h at 37°C with constant shaking, 0.5 M iodoacetamide in the same buffer was added to a final concentration of 17mM. All materials were left for 1 h at

room temperature. These extracts were collected and dialysed against four changes of deionized DW for 48 h at 4°C in dialysis tubing with 12,000 Molecular weight (MW) exclusion and finally lyophilised.

3.6. ADHESION-INHIBITION ASSAY

3.6.1. Preparation of Yeast Cells

The yeast was harvested from YNB containing 500 mM galactose by centrifugation at 2332 g for 5 min in a bench centrifuge (Megafuge 1.0, Heraeus, Sepatech) and the total number of yeasts (10^7 cells ml^{-1}) were counted with an improved Neubauer haemocytometer.

3.6.2. Preparation of Epithelial Cells

Buccal epithelial cells were collected from a single donor who was 26 years old healthy male, nonsmoker and had had no recent antibiotic therapy, by gently swabbing the inside of the cheeks with a sterile swab. The swab was agitated in 6 ml PBS in a universal. The buccal epithelial cells were harvested by centrifugation at 2332 g for 5 min in a bench centrifuge (Megafuge 1.0, Heraeus, Sepatech) and washed twice in 5 ml PBS to remove loosely-bound microorganisms. After the second washing the epithelial cells counted and adjusted to 10^5 cells ml^{-1} using an improved Neubauer haemocytometer. Buccal epithelial cells were always collected at the same time of day to minimize variability.

3.6.3. Assay for Inhibition of Adherence

The method used was based on that described by Douglas *et al.* (1981). Buccal epithelial cell suspensions (1 ml containing $1x10^5$ cells ml^{-1}) were centrifuged and the supernates discarded. Mannoproteins adhesin (1 ml) containing 1 mg ml^{-1} protein was added to the epithelial cell pellets and mixed using a whirlimixer. The mixtures were

incubated for 30 min at 37°C on an orbital shaker. After this treatment, the epithelial cells were recovered by centrifugation, resuspended in PBS (1 ml) and used for adherence assay with *C. albicans*.

The adherence assay of Douglas *et al.* (1981) was used. The epithelial cells (0.1 ml of 10^5 ml^{-1}) and the yeast were mixed in small bijoux bottles and incubated at 37°C with gentle shaking for 45 min. Control mixtures were set up containing epithelial cells and PBS. After incubation, 2 ml PBS was added to each bottle to stop any further attachment by dilution. The epithelial cells were collected on polycarbonate filters (12 μm pore size; 25 mm diameter; Costar Nuclepore, UK), which retains epithelial cells but allows free yeast cells to pass though. The sample was washed with 30 ml of PBS to remove unattached yeasts. The washed epithelial cells on the filters were placed on labelled slides and left for a few minutes to air dry. Epithelial cells on the filters were fixed with absolute methanol and stained by the Gram procedure. After drying, filters were mounted under coverslips using DPX mountant (BDH Chemicals Ltd). The markers on the slides were covered and the slides mixed to avoid personal bias. The number of adherent yeasts on each of 100 epithelial cells was counted on each filter under 400x magnification. Three filters were prepared for each assay and all adherence assays were performed in triplicate.

3.7. IDENTIFICATION AND QUANTITATION OF LECTIN-LIKE ADHESINS ON *CANDIDA ALBICANS*

3.7.1. Fluorescein-Labelled-Neoglycoproteins

Fluorescein-labelled neoglycoproteins (glycosylated serum albumin) containing sugar residues were bought from Sigma (Table 5). These reagents which were used as probes, were obtained by allowing phenylisothiocyanate glycosides to react with serum albumin and labelled by reaction with fluoresceinylisothiocyanate as described by Monsigny *et al.* (1984). All products of neoglycoproteins were supplied freeze-dried.

Table 5. Sugar and FITC contents of neoglycoproteins: Mol ratio per mol bovine serum albumin from Sigma catalogue

Neoglycoprotein	Abbreviation	Mol ratio per BSA		
		sugar	FITC	Catolog No
α-D-glucopyranosyl-FITC-albumin	Glu-BSA-FITC	18.0	2.6	A 5543
α-D-galactopyranosyl-FITC-albumin	Gal-BSA-FITC	18.0	1.8	A 2420
α-L-fucocopyranosyl-FITC-albumin	Fuc-BSA-FITC	16.0	2.3	A 5793
β-D-lactcosylated-FITC-albumin	Lac-BSA-FITC	15.9	1.6	A 8040
α-D-mannosylated-FITC-albumin	Man-BSA-FITC	17.0	2.0	A 7790
(no sugar) FITC-albumin	BSA-FITC		11.0	A 9771
Albumin-1-amido-1-deoxy-L-fucose	Fuc-BSA	20.0		A 6033
Albumin-2-amido-2-deoxy-D-galactose	Gal-BSA	15-25.0		A 5960

For use, the neoglycoproteins were weighed and diluted in the appropriate buffers. The probe solutions were centrifuged in the microcentrifuge (1166 g for 5 min) to removed undissolved probe and the supernates used for labelling.

3.7.2. Fluorescence Microscopy

3.7.2.1. Equipment and method

Fluorescence microscopy was done with a Oberkochen Axiophot Photomicroscope (Carl Zeiss D-7080), fitted with Blue H 485 and exciter filter BP 485/20, a chromatic beam splitter FT 510, a barrier filter LP 520 and at magnifications of 400x and 1000x. The photographs were taken with an automatic camera. The film (Kodak T MAX 400) used was black and white which was kindly obtained by Mr. A. B. Ellis (IBLS, Division of Infection and Immunity, University of Glasgow).

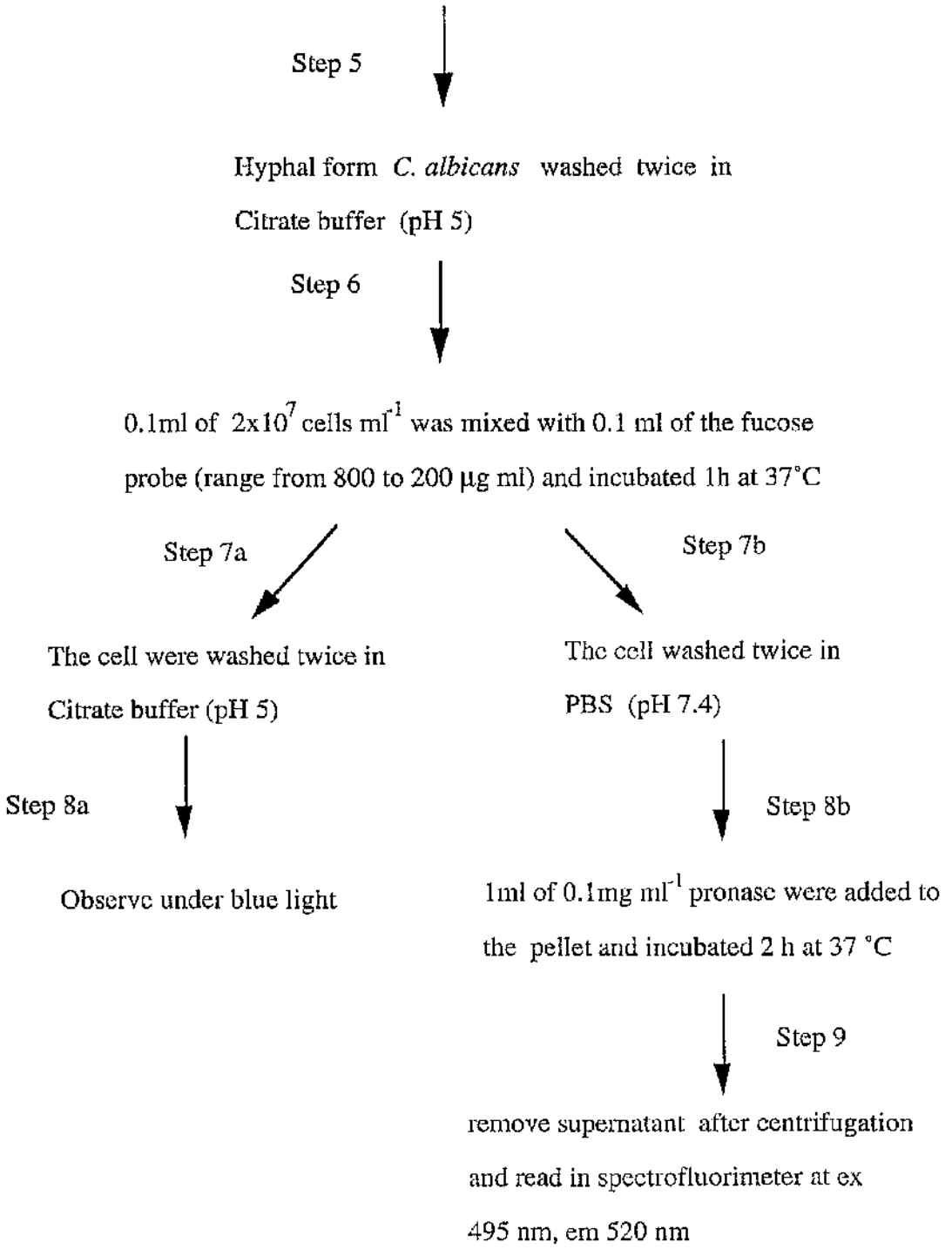
The yeast cells were grown in YNBGal and also in MLMwAA were labelled with neoglycoproteins. Only 1 ml of 300 μg ml^{-1} of Fuc-BSA-FITC probe and $1x10^7$yeast cell ml^{-1} were used as final concentrations. The hyphal-form cells (2.5 h, 4 h) in 199 Medium and (4 h) in GG were labelled using the same procedure (Figure 7). After washing, the cells were resuspended in 0.2 ml of 0.1 M citrate buffer at pH 5.

3.7.3. Fluorescence Microscopy with Image Analysis

3.7.3.1. Equipment and method

A Vickers M17 microscope was fitted with a dichroic mirror which reflects wavelengths below 514 nm and transmits those above 540 nm. The fluorescence output was passed to a Falcon LTC 1162 ISIT very low light TV camera (Custom Camera Designs Ltd. Well Somerset, UK) which was controlled through a camera control type CCU 1595 (Custom Camera Designs Ltd. Well Somerset, UK) with the automatic gain control replaced by a fixed manual gain control (type RCU 15510). There were two displays, one was on a monitor attached to a computer and printer (Star LC24-200) and

Figure 7. Flow chart of standard protocol for exploring the binding of the fucose probe to the hyphal-form cells. The figure is a continuation of Figure 6



the other was a video display (Philips monochrome) connected to a video printer (Sony UP-850). A schematic drawing of the equipment is in Figure 8.

The fluorescence signals were collected by the video camera and the video images transferred to the Compaq 386 computer through a Matrox frame digitiser, the camera setting being kept constant. All commands were typed to the computer monitor (Figure 8).

Semi-quantitation was done using two computer programs measuring large and small scales. For visualization of the hyphal-form cells, the samples were labelled with one of 5 neoglycoproteins (fucose, mannose, galactose, glucose, lactose) according to section 3.7.2.1 and viewed on the video display under bright light at 1000x. The image of each cell was saved from the display to a file on the hard disk. This procedure was then applied to the same cell but under blue light.

For quantitative analysis of the image of each fluorescence probe on the yeast and germ tube portions of the hyphal-form cells, the signals were output from the computer to a high resolution black-and white-monitor. Two computer programs were used to display, capture and measure the fluorescence. Some images were recorded on large-scale as 100x100 pixels on the screen, while others on a small-scale had 10x10 pixels.

Large-scale experiments: The hyphal-form cells were labelled with the fucose probe. After the yeast cell portion had been selected under bright light, a cell was captured in the area which had previously been created on the screen (Figure 9). Then the light was changed to blue without shifting the field of microscopy, and the fluorescence intensity of the yeast cell portion area was captured and measured. The area was 6.7 μm^2 which corresponded to 10,000 pixels. In the next step, the fluorescence intensity of the germ tube portion area of the same hyphal-form cell was measured similarly. The fluorescence intensity of portions of ten cells was measured by alternation process. The fluorescence was recorded and analyzed initially through a program written in the Pip-ez software supplied by Matrox (Dorval, Quebec, Canada) and later through a program written by Dr. A. T. J. Dow, (IBLS, Division of Infection

Figure 8. Schematic drawing of the equipment for image analysis (based on Shotton, 1993)

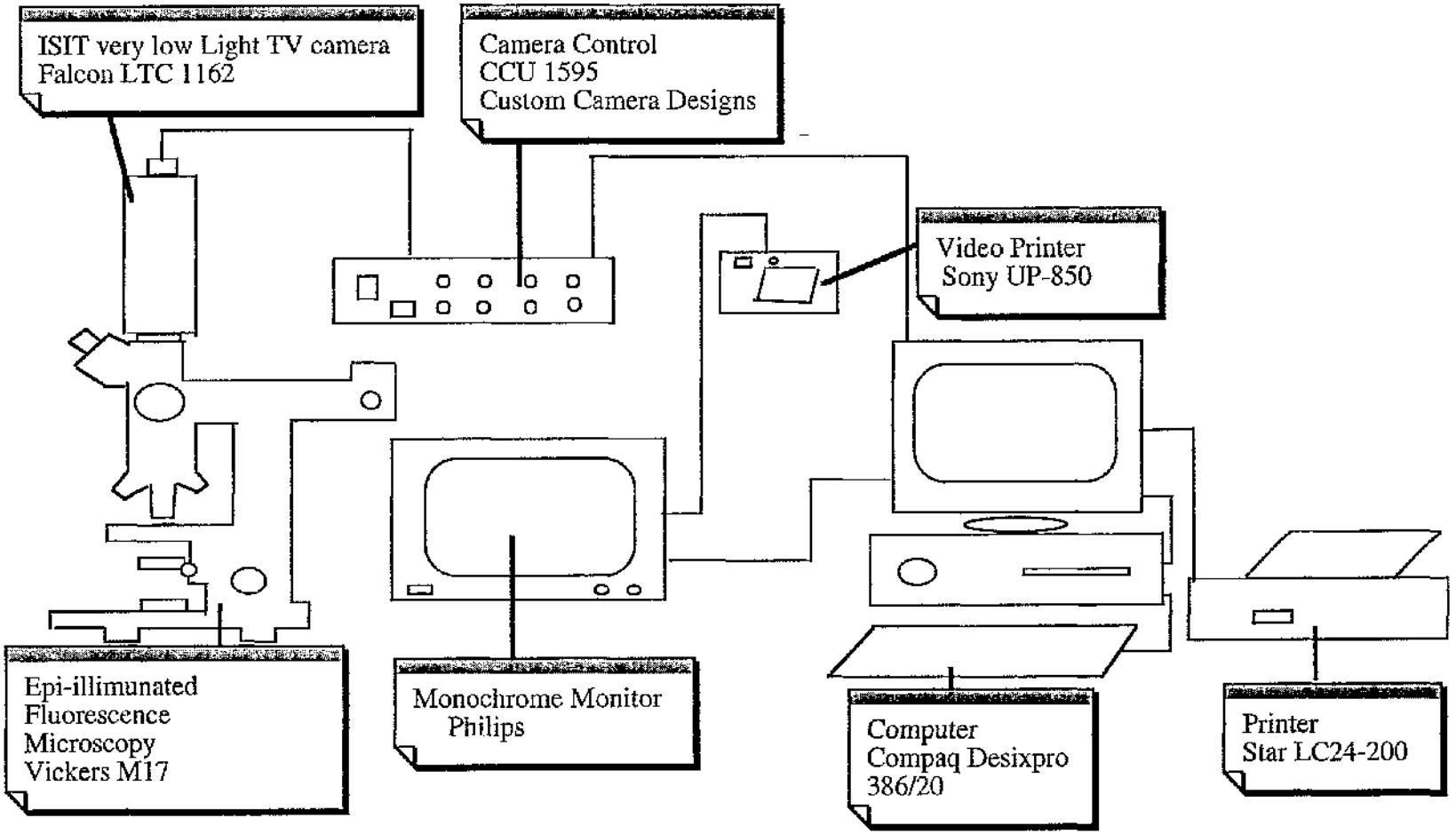


Figure 9. Plan for taking measurements of fluorescence intensity in 4 different areas on and around the hyphal-form *C. albicans*

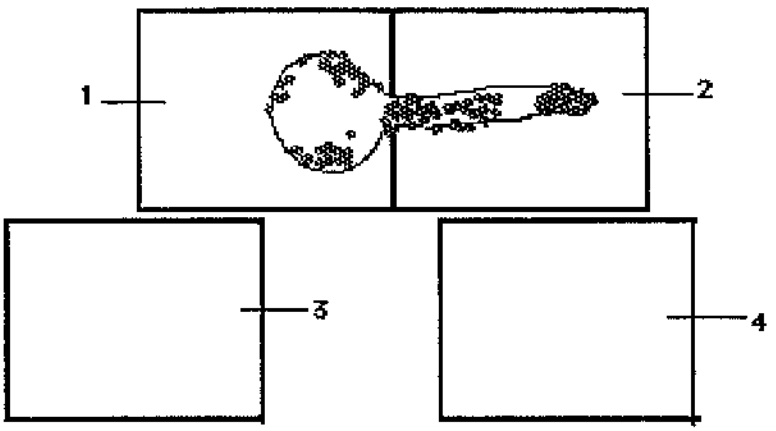
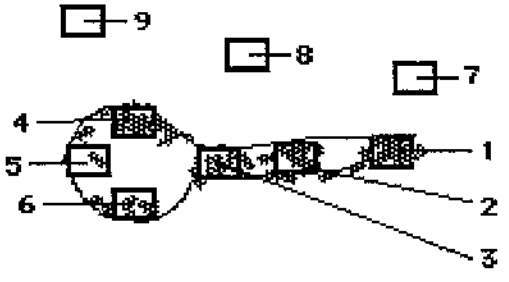


Figure 10. Plan for taking measurements of fluorescence intensity in 9 different areas on and around the hyphal-form *C. albicans*



and Immunity, University of Glasgow) and used afterwards by Wójciak and Crossan (1994). This procedure was repeated twice on two separate days.

Small-scale experiments: The fluorescence measurement system was used for capturing and storing the cells both under bright and blue light on the computer hard disk before being subjected to analysis. For each experiment, 30 cells were copied to the hard disk using the Semper program. This procedure was repeated on three days. The fluorescence intensity was then recorded in 0.67 μm^2 area and corresponded to 100 pixels using the partition command of the Semper program. Scanning began at the tip end of the germ tube portion of the hyphal-form cells (Figure 10). Similar measurements were done with different areas on the yeast cell portion of the hyphal form cells.

Fluorescence intensity of the yeast cell and the germ tube portions of the hyphal form cell, labelled with 5 probes, was measured. The experiments were carried out on the same day's preparation of *Candida albicans*. The hyphal-form cells under bright light and blue light for each probe were captured and copied onto hard disk. The experiment was repeated on two days, each time with 10 of the hyphal-form cells for each probe being stored. To minimize quenching problems, measurement of the fluorescence intensity of the probes was started from the lactose probe and continued in the order galactose, glucose, mannose, fucose.

3.7.3.2. Presentation and analysis of results

From the hard disk records, the cells under bright light and blue light were displayed. Using each cell under blue light, a contour map was drawn using the contour command, and the pixel intensity of the cell was plotted as surface height in a line drawing using the y-modulus command. Four images of the cell were displayed on the screen and printed out on high density paper (Sony Type II UPP-110 HD).

Specific images were divided into individual image points (pixels). Each pixel consisted of a number ranging from 0 (dark) to 256 (bright) according to the intensity of transmitted light or grey level at that point. The digital image consisted of a 100x100

matrix of picture elements for large scale and 10x 10 for small scale. Large scale experiments were presented as the sum of fluorescence intensities of each portion of the cells and of their backgrounds. Interpretation of small scale experiments was done with a different statistical program. Boxplots were drawn for each individual small square representing a total of 90 cells from all experiments. Each boxplot shows the truncated range, median, quartiles, 95 % confidence intervals and outlier values. A bar chart was also presented for the truncated mean and SEM using the Minitab Statistics package on a Macintosh computer. Students' t-test was used to determine the significance of difference in fluorescence intensity between the yeast cell and the germ tube portions of hyphal-form *C. albicans* images.

3.7.4. Spectrofluorimetry

3.7.4.1. Equipment and method

A fluorescence spectrophotometer (Model 2000, Hitachi, Ltd. Tokyo, Japan) with xenon lamp was used. The fluorimetry function was choosen for quantitative analysis of the probes from the main menu displayed on the screen. By setting the screen in the data mode, the fluorescence intensity of samples and their concentration was obtained. The excitation wavelength was 495±10 nm and the emission wavelength was 520±10. The final output was a standard curve from which the labelled probe concentration could be obtained.

For pH experiments, incubating buffers were prepared using 0.1M phosphate buffer pH 6.0, 7.0, 8.0, and 0.1M citrate buffer at pH 3.0, 4.0, 5.0 containing 1% (w/v) BSA. The fucose probe (Fuc-BSA-FITC) was prepared as 200 $\mu g\ ml^{-1}$ in 0.1M phosphate buffer and citrate buffer at different pHs.

In preliminary experiments, yeast cells grown in YNB containing 500 mM galactose were used after washing twice in buffers at the different pH on the bench centrifuge (5760 g, 5 min); 0.1 ml of yeast cell ($2x10^8$ yeast cells ml^{-1}) and Fuc-BSA-FITC (0.1 ml of 200 $\mu g\ ml^{-1}$) were mixed in Eppendorf tubes to give final

concentrations of 10^8 yeast cells ml^{-1} and 100 µg probe ml^{-1} respectively. All preparative procedures were done under sterile conditions. The sample tubes were wrapped with aluminium foil to prevent quenching and incubated at 37°C for 1 h with rotory shaking (150 rpm). Afterwards, the cells were pelleted using the microfuge (2380 g, 10 min) and the supernate removed. The pellets were washed in PBS at pH 7.4 and finally resuspended in 1 ml of PBS at pH 7.4 containing 0.1 mg ml^{-1} of pronase E (Protease Type XXV, Sigma P-6911) and incubated for 2 h at 37°C to release the fluorescein probe. The treated cells were centrifuged on the microfuge (2380 g 10 min) and the supernate was removed and read in the spectrofluorimeter.

To determine the proper incubation time for binding of the fucose probe to the yeast cells, a range from 15 to 120 min was tested. All samples were pretreated with pronase E as describe above. For the saturation curve, the fucose probe was diluted in 0.1 M citrate buffer at pH 5.0 to give concentrations from 3.1 to 400 µg ml^{-1}.

The hyphal-form cells ($1x10^7$ cells ml^{-1}) were labelled with 200 µg probe ml^{-1} of the fucose probe into 0.1 M citrate buffer at pH 5.0 as final concentration. After washing the cells with PBS at pH 7.4, pronase E (4000 unit g^{-1}.) was added and incubated at concentrations of 1.0, 0.5 and 0.1 mg ml^{-1} to remove the probe from the cells.

To explore the effect of the concentration of hyphal-form *C. albicans*, cells with germ tubes were prepared between $2x10^4$ and $2x10^8$ ml^{-1} in 0.1 M citrate buffer at pH 5.0 and reacted with 200 µg ml^{-1} of Fuc-BSA-FITC in the same buffer. The final concentration of the cells was between 10^4 and 10^8 ml^{-1}, and the probe final concentration was 100 µg ml^{-1}.

Using hyphal-form *C. albicans* ($1x10^7$ cells ml^{-1}), the effect of pH on the binding of five neoglycoproteins labelled with FITC (100 µg ml^{-1}) was investigated as done in the yeast cells binding procedure. The effect of incubation time on the binding of Fuc-BSA-FITC was likewise explored.

A standard curve for the fucose probe with and without treatment with pronase was carried out. The stock solution was prepared in 0.1 M phosphate buffer pH 7.2

final concentration as 2000 μg ml^{-1}. The probe 0.1 ml was dissolved in 0.9 ml of 0.1 M phosphate buffer pH 7.2 and 0.1M citrate buffer pH 5.0 to give final added concentration of 200 μg ml^{-1}. The probe solutions (0.1 ml) were diluted in 1 ml PBS pH 7.4 containing 0.1 mg ml^{-1} pronase. Two controls of the probe solutions were prepared in 1 ml 0.1 M phosphate buffer pH 7.2 and 0.1 M citrate buffer pH 5.0 without pronase. After incubation for 2 h at 37°C, the fluorescence intensity was recorded.

Standard curves of five different probes: fucose, mannose, glucose, galactose, lactose), consisting of neoglycoproteins labelled with FITC, were dissolved and serially diluted over the range 0.3 to 40 μg ml^{-1} in 0.1M citrate buffer pH 5.0 and assayed in the spectrofluorimeter. The hyphal-form cells ($1x10^7$ cells ml^{-1}) were also labelled with these probes (final concentation 200 μg ml^{-1}) as before. Concentration of these probes which bound to the cells were calculated using their standard curves.

Production and non-production of germ tubes by yeast under different conditions was investigated after washing the starved cells (section 3.3.2.2) twice in DW . The cells were suspended in 15 ml 199 Medium (pH 4.0) and 30 ml of 199 Medium (pH 6.7) final concentration $1x10^7$ cells ml^{-1} in a universal. The medium was divided into the universal in 3 ml portion cultures. The Medium (pH 6.7) cultures were incubated up to 5 h at 37°C and 22°C, and in Medium (pH 4.0) at 37°C. Incubation of yeast cells was started from 5 h, and each hour new yeast cell suspension was incubated at 37°C to allow production of germ tubes. Each universal was kept on ice before incubating the cells with the probes. The cell numbers were counted and adjusted to $2x10^7$ ml^{-1}, and germ tube production was recorded by counting 5 fields at 1 h intervals up to 3 h. The labelling procedure was applied as before.

The effect of 2 mM Ca^{++} and Mg^{++} on the binding of Fuc-BSA-FITC was investigated in 0.1 M citrate buffer without Ca^{++} and Mg^{++}, and 0.1 M acetate buffer with and without Ca^{++} and Mg^{++} at pH 5.0 ; 0.1 M phosphate buffer without Ca^{++} and Mg^{++} and 0.1M tris-HCl buffer with and without Ca^{++} and Mg^{++} at pH 7.2. The

hyphal-form cells (final concentration $1x10^7$ cells ml^{-1}) were mixed with 0.1 ml of the probe (final concentration 100 μg ml^{-1}).

3.7.4.2. Presentation and analysis of results

Measurement of fluorescence intensity of neoglycoprotcins which had been bound on the cells was displayed on the screen and printed on paper. Fluorescence intensity of the probes was expressed in arbitrary units (AU). The measurement screen showed the standard curve from which was generated the concentration of neoglycoproteins which had been bound on the cells.

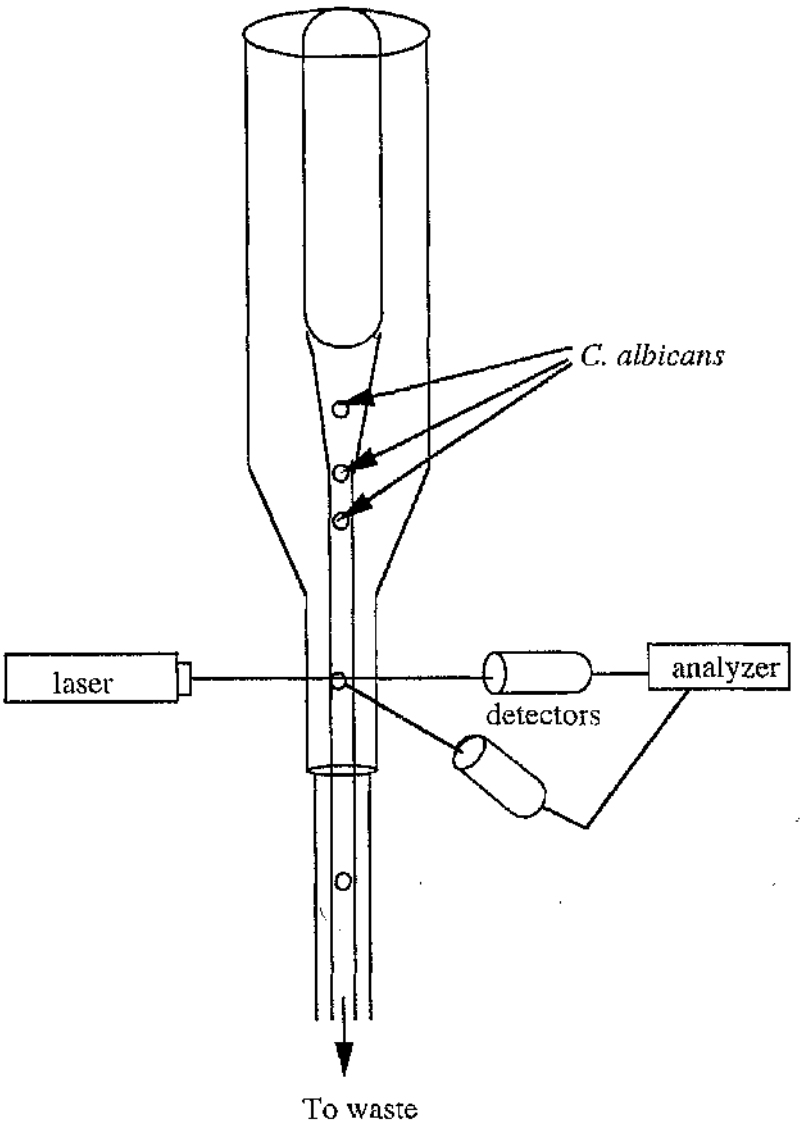
3.7.5. Flow Cytometry

3.7.5.1 Equipment and method

A flow cytometer, FACScan; Becton Dickinson Immunocytometry System (BDIS), equipped with a standard phycoerythrin-fluorescein isothiocyanate filter set, forward and side light scatter detectors, and computer analysis program (H. P. Consort 30) was used for flow cytometric analysis. The schematic drawing of the equipment is in Figure 11. The laser was a class 3 B with an emission at 488 nm and an output of 250 mW. Fluorescence was calibrated using caliBRIDE beads of known fluorescence intensity (BDIS Ltd, Abingdon, UK) and analysed by Auto COMP software. Cells size was also calibrated according to the contour plot appearance of yeast and hyphal-form cells (Lynch *et al.*, 1993) and this setting program was used throughout all experiments. Lysis II is an integrated program software which was used to acquire and analyze data from the flow cytometer. This equipment was sited in the Department of Immunology, Faculty of Medicine, University of Glasgow).

The probe-labelled *C. albicans* were analyzed by forward and side light scattering and by fluorescence. Light scatter patterns of the cells during germ tube production by flow cytometry (FCM) were analysed to determine cell size by forward scatter light scatter (FSC) and granularity of internal structure (cytoplasmic intricacy and number of nuclei) by side light scatter (SSC). The starved yeast cells were

Figure 11. Schematic drawing of the operating principle of the flow cytometer (based on Shapiro, 1990)



incubated in 199 Medium for up to 2 h to produce germ tubes. The cells were examined for germ tube production under light microscopy at 1 h intervals and collected at different times for analysis by flow cytometry. For this purpose the cells were diluted 1:10 in 0.1M citrate buffer pH 5.0. The cell suspension at approximately $2x10^6$ ml^{-1}, was mixed with a pipette and immediately subjected to flow cytometry to avoid aggregation.

Binding of the neoglycoproteins-FITC during germ tube production was tested by this method. Labelling of the cells was performed as previously described for spectrofluorimetry except that pronase treatment was omitted. Briefly, germ tubes were produced for up to 2 h, according to section 3.3.3.1, to avoid aggregation of the cells. The hyphal-form cells (final concentration $1x10^7$ cells ml^{-1}) were labelled with the fucose or lactose probes (final concentration 100 µg ml^{-1}) in citrate buffer pH 5.0 for 1 h at 37°C. The pellets were washed twice in 0.1 M citrate buffer pH 5.0. The final suspensions were diluted 1 : 10 and dispersed as thoroughly as possible by repeated mixing with a 1 ml pipette in a polystyrene tube (Falcon, 4 x 0.5 cm). This was necessary to minimize cell clumping which would have blocked the flow cytometer and also given false readings. The reading was taken as rapidly as possible after mixing and the results presented as a two dimensional distribution analysis or as a histogram. All measurements of flow cytometry fluorescence are in arbitrary units (AU). Optimum settings were obtained at the outset of the project, and these were used throughout in order to standardize the results. Unlabelled cells were used as standard negative reference cells. This procedure was repeated on two days for a total of 3,000 and 5,000 cells respectively. Later, the measurements were done using 3000 cells.

To compare binding of the fucose probe by the cells grown in 199 Medium (pH 6.7) at 37°C, 22°C and 199 Medium (pH 4.0), the section 3.3.3.1 method was applied. Production of the hyphal-form cells was observed and counted under bright light microscopy at 1 h intervals and analyzed by FCM to produce light scatter patterns. After labelling the cells with the fucose probe, fluorescence intensity was determined by FCM.

To examine different media for expression of lectin-like adhesins specific for the fucose probe, the starved cells were incubated in 199 (section 3.3.3.1) and Glucose-Glycine Media (section 3.3.3.2) for up to 2 h at 37°C to produce germ tubes. This experiment was done as previously, but only the fucose probe was used for labelling the cells. Yeast cells grown in YNBGal (section 3.3.2.1) and the hyphal-forms grown in 199 Medium (section 3.3.3.1) were labelled with neoglycoproteins-FITC (fucose, mannose, glucose, galactose, lactose) as mentioned before.

The effect of Ca^{++}, Mg^{++} and different buffers on binding of the fucose probe was done by adding 4 mM Ca^{++} and Mg^{++} (final concentration 2 mM) to the buffers which were 0.1 M acetate (pH 5.0) and 0.1 M tris-HCl buffer (pH 7.2). As a control, 0.1 M acetate (pH 5.0), 0.1 M tris-HCl (pH 7.2), 0.1 M citrate (pH 5.0) and 0.1 M phosphate (pH 7.2) buffers without Ca^{++} and Mg^{++} were used. The hyphal-form cells (0.1 of $2x10^7$ cells ml^{-1}) were suspended in the buffers and labelled with the fucose probe (0.1 of 200 µg ml^{-1}). The cell suspension was prepared in 10 mM EDTA (final concentration 5 mM) as chelator in 0.1M tris-HCl pH 7.2. Labelling of hyphal-form *C. albicans* with the fucose probe was done at different temperatures (37°C, 22°C and 4°C.) for 1 h.

Comparison of Fuc-BSA-FITC, Lac-BSA-FITC and BSA-FITC (final concentration 100 µg ml^{-1}) was done with the hyphal-form cells. Fuc-BSA-FITC and Lac-BSA-FITC were used as positive and negative controls respectively. To explore the specificity of Fuc-BSA-FITC binding to the hyphal-form of *C. albicans*, Fuc-BSA and Gal-BSA without FITC (Sigma) and L-fucose (Sigma) and D-galactose (BHD) were used. The hyphal-form cells (0.1 ml of $3x10^7$ cells ml^{-1}) were mixed with the neoglycoproteins without FITC (0.1 ml of 6 mg ml^{-1}) and the sugars (0.1 ml of 75 mg ml^{-1}) and incubated 10 min at 37°C and then the fucose probe (0.1 ml of 600 µg ml^{-1}) was added to each solution and incubated 1 h as in previous experiments. For the positive control, the hyphal-forms were labelled by the fucose probe; the negative control was unlabelled cells.

C. albicans wild type (strain MEN) and its germ tube-deficient mutant (strain MM2002) were analysed after labelling with Fuc-BSA-FITC. For production of germ tubes, starved cells of the strains were inoculated into 199 Medium as described in section 3.3.3.1 and the cells suspension was counted at 1 h intervals for germ tube production. The strains were labelled by the fucose probe for comparison of fluorescence intensity. Unlabelled cells were used as the negative control.

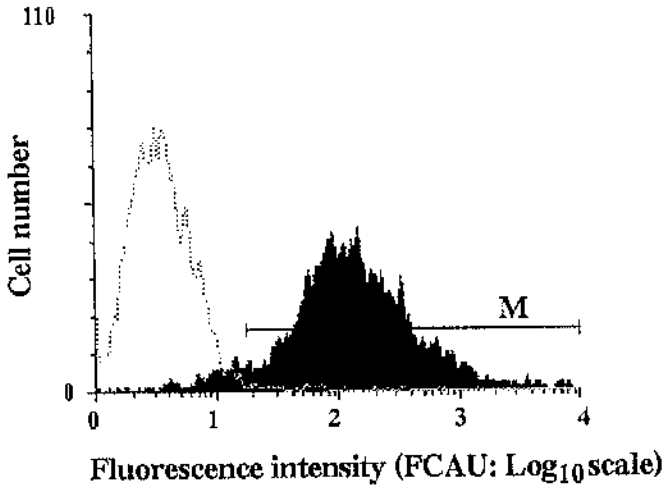
3.7.5.2. Presentation and analysis of results

Fluorescence intensity of the unlabelled and labelled cells with the probes were recorded by Lysis II, Becton-Dickinson Immunocytometry System (BDIS) and stored on a floppy disk (Scottish H.E., DS high density 2 MB). All these data were analysed by the Lysis II program.

Dual parameter contour plots were used to distinguish and define the morphologic forms of *C. albicans*. Forward scatter, FCS, (cell size) and side scatter, SSC, (granularity of internal structure) patterns were used. These contour plots provided a series of isometric lines that are intended to show the number cells at selected levels.

The fluorescence intensity of the cells was measured on a logarithmic scale which appears on the ordinate, while the relative number of the cells appears on the axis. Grey lines indicated background of non-labelled cells. For the clearer presentation of the histograms, the background was subtracted and a marker set created starting from Log_{10} 1.76 left channel to 4.0 right channel by the Lysis II program (Figure 12). Mean fluorescence intensity of the cells and percentage of the total cells in the marker set was compared to the whole histogram.

Figure 12. Schematic drawing for interpretation of flow cytometry histogram by selecting marker boundaries. The line on the histogram (M) indicates these boundaries. The grey line represents unlabelled cells and the black area the cells labelled with the fucose probe



3.8. IDENTIFICATION OF FIBRONECTIN ADHESINS ON *CANDIDA ALBICANS*

3.8.1. Preparation of Colloidal Gold

Collodial gold particles of 20 nm diameter were prepared by the controlled reduction of an aqueous solution of chloroauric acid using sodium citrate as reducing agent, as described by Geoghegan and Ackerman (1977). All solutions were prepared in deionized, double DW and were passed through a 0.2 μm microfilter. Two ml of 1% (w/v) chloroauric acid was added to 198 ml of deionized, double-DW to give a final concentration of 0.02% (w/v) in a clean siliconized Ehrlenmeyer flask. A fresh solution of 1% (w/v) aqueous sodium citrate (6 ml) was rapidly poured into a boiling solution of the cholroauric acid under vigorous mixing. As the solution boiled it turned dark blue and was then heated under reflux until the typical wine-red colour of colloidal gold was reached, after which it was cooled to 4°C. Before use, the colloid was centrifuged at 800 g (MSE Chilspin) for 10 min in order to remove aggregates and the supernate kept at 4°C. The number of particles per unit volume was calculated spectrophotometrically at A_{520} as described below.

3.8.2. Fibronectin-Coated Colloidal Gold

Studies on the adsorption of macromolecules to the colloidal gold particles have shown that it is pH and concentration dependent (Geoghegan and Ackerman, 1977). The gold probe concentration was adjusted to $A_{520} = 1.0$ equivalent to approximately 7×10^{11} particles ml^{-1} De Mey (1986). For protein adsorption, the pH of the gold particle preparation was adjusted to 7.0 with 0.2 M potassium carbonate. Two drops of 1% (w/v) polyethylene glycol (PEG, Sigma) M.W. 20,000 were mixed with the gold particle suspension (2 ml) in a conical plastic centrifuge tube to stabilize it before the pH electrode was inserted.

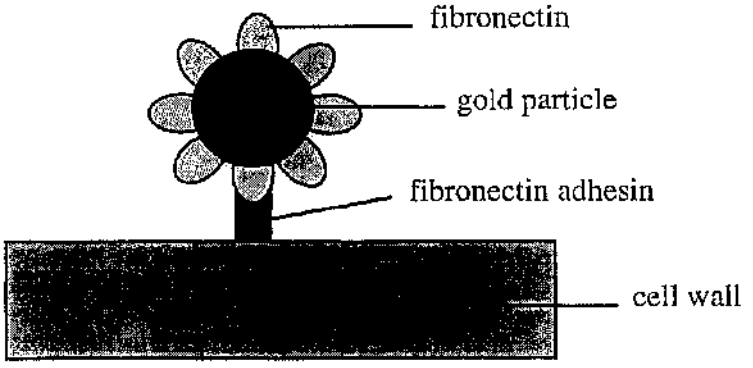
Fibronectin coated gold colloid was prepared according to Pescioata Peters and Mosher (1987) by incubating with plasma fibronectin (Figure 13). In order to

determine the appropriate amount of fibronectin for coating the colloidal gold, a saturation test (Horisberger and Rosset, 1977 and modified by Dr. L. Tetley, IBLS, Division of Infection and Immunity, University of Glasgow) was done. First the human plasma fibronectin was checked for purity by SDS-PAGE (Appendix 7.1 and 7.2).

Fibronectin dissolved as 2 mg ml^{-1} in PBS was dialyzed against 2 mM tris-HCl containing 15 mM NaCl (pH 7.4) at 4 °C with four changes. The solution was then diluted 1: 8 in the same buffer. A dilution series was made in a multiwell plate (Appendix 7.3). Fibronectin, the buffer and gold colloid were mixed and left for 2 min to complex fully, after which 50 µl of 10% (w/v) NaCl was added. After 30 sec agitating the plate, flocculation was observed as a change from the wine red colour to purple-blue. Fibronectin solution (20 µl) stabilized the gold against salt-induced floculation. From the results, an increased volume of fibronectin solution was calculated to prepare the final fibronectin gold probe (Appendix 7.3).

Fibronectin solution was added to gold colloid whilst the latter was stirred rapidly. There was a slight colour shift during the almost instantaneous complex formation. After 2 min stirring, unbound areas on the gold particles were stabilized with 1% (w/v) polyethylene glycol (PEG) pH 6.0 in deionized, double DW (final concentration 0.05%). Gold colloidal coated with fibronectin (Au_{20}-Fn) was centrifuged at 28000 g (MSE Height speed) for 30 min at 10°C. At the end of centrifugation, three phases are obtained; a clear supernate containing free fibronectin; a dark red sediment at the bottom, which corresponded to the fibronectin-gold complex; and a black spot that remained along the side near the bottom of the tube as gold not stabilized by the fibronectin and PEG. The supernate was carefully aspirated as completely as possible and discarded. Fibronectin-coated gold particles were recovered and resuspended in 2 ml of 2 mM tris-HCl (pH 7.4) buffer containing 0.005% (w/v) sodium azide and kept at 4°C overnight. The particles were centrifuged at 28000 g for 30 min at 10°C once on the following day. After resuspension with 2 mM tris-HCl

13. Schematic drawing of *C. albicans* cell wall labelled with a single fibronectin-coated collodial gold (based on Benhamou, 1989)



containing 15 mM NaCl at pH 7.4, Fibronectin-coated gold particles were adjusted by spectrophotometer to give $A_{520} = 1.0$. The same labelling method was used for preparing PEG-coated particles as a control.

3.8.3. Electron Microscopy

3.8.3.1. Equipment and method

A EM 902 Transmission electron microscope (Zeiss, Germany) was used. *C. albicans* grown into YNBGal and MLMwAA according to section 3.3.2.1 and 3.3.2.2 and the hyphal-form cells produced in 199 Medium according to section 3.3.3.1 was labelled with fibronectin-coated gold particles (Au_{20}-Fn). The yeast-form cells ($2x10^8$ cells ml^{-1}) were resuspended in 2 mM tris-HCl (pH 7.4) buffer containing Ca^{++} and Mg^{++} (2 mM) and washed twice in the same buffer. The cells were fixed with 1 ml of 2.5% (w/v) paraformaldehyde (BDH) which was prepared fresh in PBS at pH 7.2. The cells were incubated at 4°C for 30 min and centrifuged for 5 min at 3500 rpm and then mixed with 1ml of 0.2 M glycine (Sigma) in PBS at pH 7.2 at 4°C for 10 min. The cell pellet was spun for 5 min at 3500 rpm and washed once in 2 mM tris-HCl (pH 7.4) buffer containing Ca^{++} , Mg^{++} (2 mM) and 1% (w/v) BSA. The pellet was resuspended in 0.1 ml of the same buffer and mixed with 0.1 ml of Au_{20}-Fn. After incubation of the mixture at 22°C for 30 min, the cells were centrifuged and washed twice in 1 ml of same buffer. The specimen was prepared for Transmission Electron Microscopy as described in Appendix 7.4.

3.9. BIOCHEMICAL METHODS

3.9.1. Estimation of Carbohydrate

Carbohydrate was estimated by the method of Dubois *et al.* (1956) with mannose as a standard. In this procedure, 0.05 ml of 80% phenol (v/v) was added to the sample

containing 0-100 μg ml^{-1} carbohydrate. Reagent grade concentrated sulphuric acid (5 ml) was rapidly added and the samples were left for 30 min at room temperature to allow colour development before reading at 485 nm against a blank of DW in a spectrophotometer.

3.9.2. Estimation of Protein

Protein was determined using the method of Lowry *et al.* (1951) with BSA as standard. The reagents were: A. 2% Na_2CO_3 in 0.1N NaOH; B. 0.5 M $CuSO_4.5H_2O$ in 1% (w/v) sodium tartrate (separate double strength solutions mixed 1 : 1 before use); C. Folin Ciocalteau reagent diluted 1 : 1 with DW (to give a total acidity of 1.0 N), D. 50 vol. of reagent A were mixed with 1 vol. of reagent B (renewed daily). The procedure was consisted in adding reagent D (3 ml) to the sample 0.6 ml, containing 0-100 μg ml^{-1} of protein, mixing well and leaving to stand at room temperature for 10 min. Reagent C (0.3 ml) was then added rapidly with immediate mixing. After a further 30 min at room temperature, the tests were read against a reagent blank in a spectrophotometer at 750 nm.

3.9.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Fibronectin (Sigma) and marker proteins (Sigma, SDS-6H) were resolved on SDS-polyacrylamide gels by electrophoresis as described by Laemmli (1970). Electrophoresis was done on a BRL (Bethesda Research Laboratory) vertical gel system with separating and stacking gels contained 7.5% (v/v) and 4.5% (v/v) acrylamide respectively. The thickness of the gel was 0.15 cm. Gels and electrode buffers contained 0.1% (w/v) SDS. Gels were prepared in glass moulds 8.0 x 8.0 x 0.3 cm and electrophoresis was done at room temperature at a constant current of 20 mA until the tracking dye had passed the stacking gel, after which the current was turned up to 30 mA until the dye reached the bottom of the gel after 4-5 hr. The gel was

stained with Coomassie brillant blue (Weber and Osborn, 1969) for 2 h - overnight and destained. Further details are given in Appendix 7.1.

The standard used to determine the molecular weight of fibronectin was SDS-6H (Sigma), included carbonic anhydrase from bovine erythrocytes (29.0k Da), egg albumin (45.0 kDa), bovine plasma albumin (66.0k Da), rabbit muscle phosphorylase B (97.4k Da), *E. coli* β–galactosidase (116.0k Da) and rabbit muscle myosin (205.5k Da). The volume of sample protein loaded into the stacking gel was 40 µl for fibronectin and 10 µl for the standard proteins.

3.10. STATISTICAL AND GRAPHICAL METHODS

The results of replicate experiments were summarized as mean ±SEM, or pictorially as boxplots with 95% confidence limits. Statworks and Minitab packages were used. Differences between means were analyzed by Student's t-test (Wardlaw, 1987).

Most of the graphs presented in this thesis were prepared using Cricketgraph on a Macintosh computer. The package Lysis II was used for flow cytometer histograms.

4. RESULTS

4.1. ADHESION OF *C. ALBICANS* GERM TUBES TO PETRI DISHES

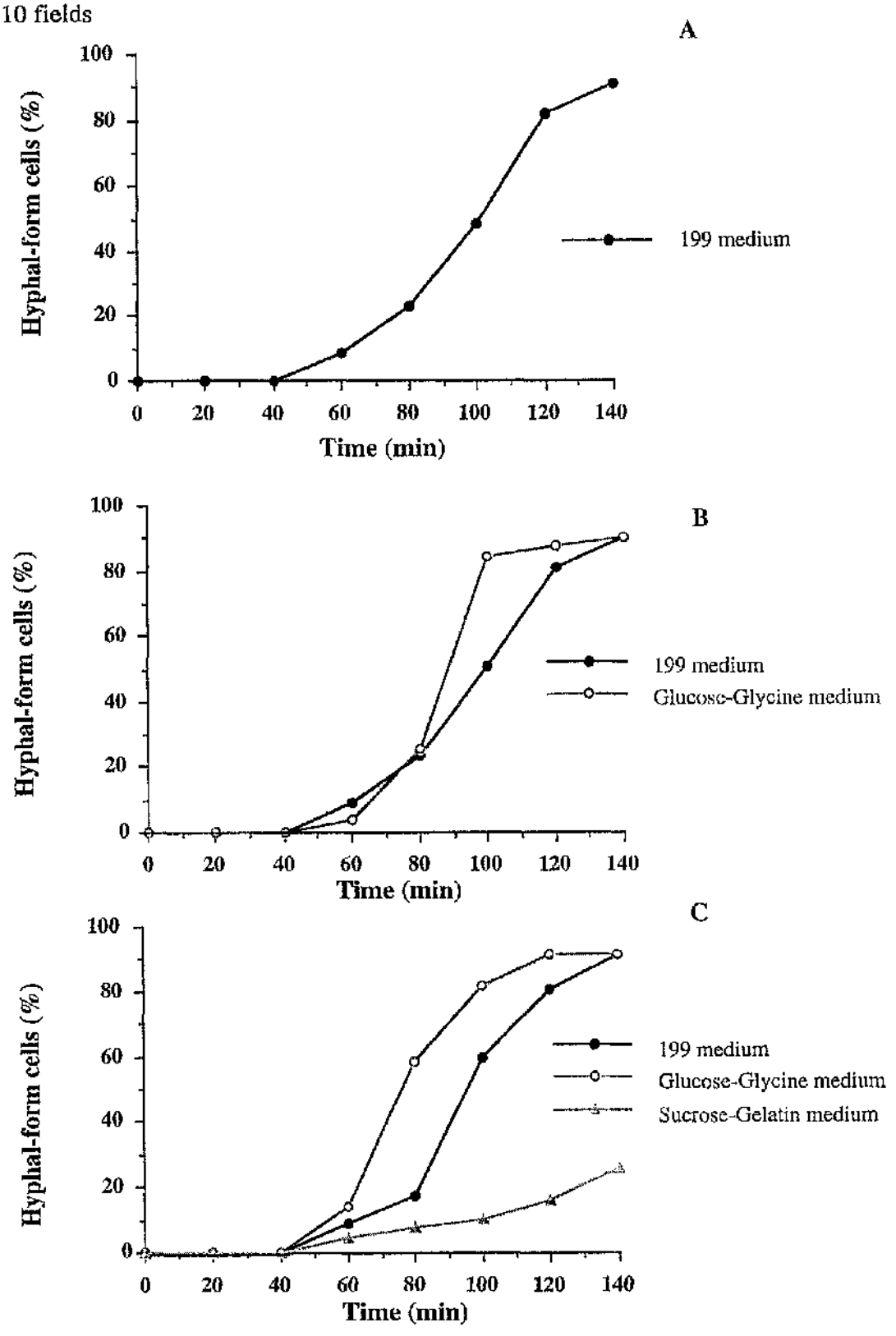
4.1.1. Germ Tube Formation by *C. albicans*

In the early stages of this investigation, experiments were made to establish satisfactory conditions for obtaining rapid and consistent germ tube production by *C. albicans.* Additionally, it was desirable to obtain germ tubes with the fibrillar layer which Tronchin *et al.* (1988) reported to contain the adhesins. These adhesins could be isolated from the surface of petri dishs by dithiothreitol treatment of the washed plastic surfaces. These investigators had described 199 Medium as giving a high percentage of hyphal-form cells within 140 min at 37°C. However before adopting this medium as a standard procedure, it was considered worthwhile to make a preliminary experiment with the Glucose-Glycine (GG) Medium of Muerkoester *et al.* (1979) and with the Sucrose-Gelatin (SG) Medium of Mayer *et al.* (1992).

In the first experiment, starved yeast cells of *C. albicans* GDH 2346 were inoculated into 199 Medium in a universal container which was incubated at 37°C on a rotary shaker at 150 rpm. At intervals of 20 min, 1 drop of the culture was removed and examined for germ tube production at 400x under bright-light microscopy. Typically, the cells in 10 fields were counted, and the number of hyphal-form cells in each field recorded. The percentage germ-tube production was thus calculated and plotted in relation to incubation time.

The three sections of Figure 14A show that for the first 40 min in 199 Medium, there was no germ tube production, but thereafter the germ tubes started to appear and reached a plateau of about 90% at 140 min. Figure 14B and Figure 14C were similar but included GG and SG Media in addition to 199 Medium. GG Medium appeared to give more rapid germ tube production than 199 Medium, whereas SG Medium gave much slower and less complete germ tube production. However, despite the faster germ tube production in GG Medium, subsequent experiments were made with 199

Figure 14. Preliminary experiments on the production of germ tubes of *C. albicans* in three different media. Each point is based on the count of 60 cells per field over 10 fields



Medium because Tronchin *et al.* (1988) had used this medium for their studies of germ-tube adhesion to plastic.

4.1.2. Production and Detection of Mannoprotein Adhesins

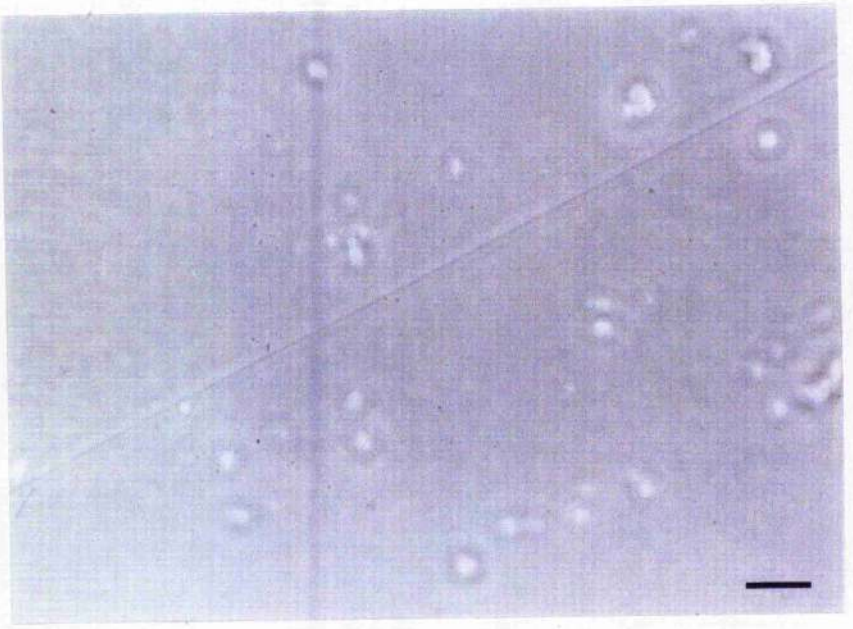
4.1.2.1. Detection of mannoprotein adhesin on petri dishes

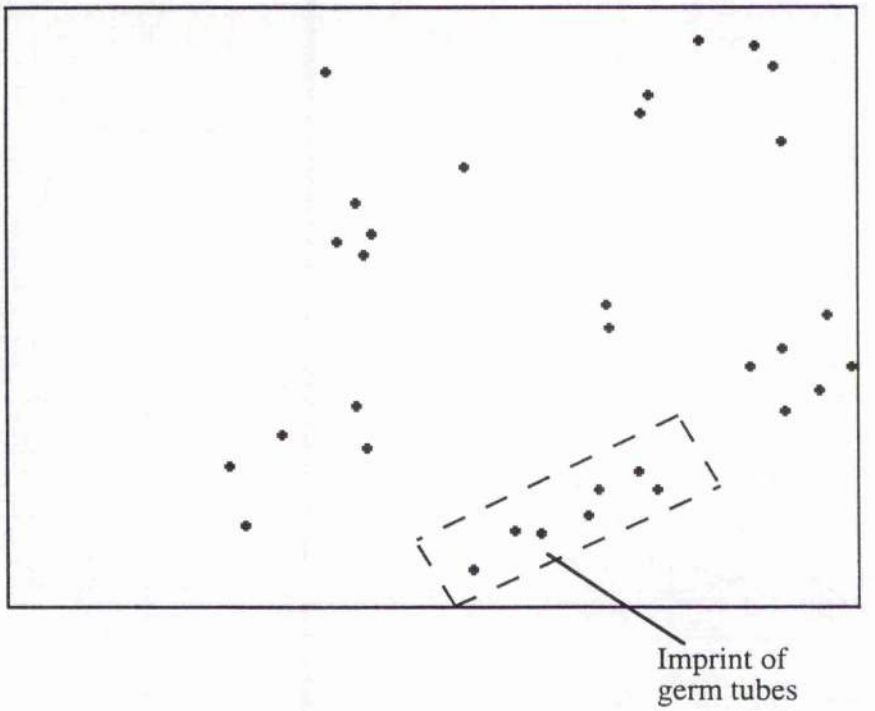
The production of mannoprotein by hyphal-form *C. albicans* on plastic petri dishes was previously reported only by Tronchin *et al.* (1988) who used Con A-coated latex beads as a lectin-specific reagent for mannose. In the present work, the same method was used to produce and detect the mannoprotein adhesin (MPA) on plastic petri dishes in which germ tubes had been produced so as to leave the cell wall fibrillar layer on the surface of the plastic. This material could subsequently be scraped off and concentrated.

In four independent experiments, *C. albicans* in 199 Medium was allowed to produce germ tubes for 3 h at 37°C in three petri dishes and, in parallel, yeast cells at 22°C. The dishes were then washed three times in DW to remove yeast and hyphal-form cells, followed by a wash with PBS containing 0.5% (w/v) BSA, and incubation for 1 h at room temperature. The Con A-coated latex beads were added and, after 30 min, withdrawn, and the dish examined by bright-field microscopy at 400x. Uncoated-latex beads and Con A-treated latex beads with α-methyl-D-mannopyranoside were used, both as negative controls.

The Con A-coated latex beads were seen (Figure 15) to bind to the plastic of the petri dish which was allowed to produce hyphal-form cells, showing that mannoprotein of the cell wall of *C. albicans* had been produced during germ tube production and had remained during the subsequent scraping and washings. The beads were arranged in outlines that reflected the germ tube portion of hyphal-form cells. There was no adhesion of either the uncoated latex beads or the Con-A treated beads with α-methyl-D-mannopyranoside (not shown). The petri dish which had had produced yeast cells contained very few Con-A coated latex beads.

Figure 15. Phase-contrast micrographs of Con-A latex beads and interpretative diagram, indicating the arrangement of particles where the germ tubes adhered to the plastic petri dish. Bar represents 10μM





4.1.2.2. Production and analysis of soluble mannoprotein adhesins

In order to obtain soluble mannoprotein adhesin (MPA) for further experiments, *C. albicans* was grown in the hyphal-form on 70 plastic petri dishes in each of three separate batches. The cells were scraped off the surfaces and the dishes washed with DW as before. Mannoprotein adhesins were extracted with dithiothreitol followed by iodoacetamide. The two extracts were pooled, dialysed, freeze-dried and reconstituted with PBS.

Table 6 presents the analyses for protein and carbohydrate estimations in the three batches of soluble MPA, expressed as the yield from 10^{10} hyphal-form cells. The protein values averaged 1.64 mg, with little variation, and the carbohydrate averaged 0.32 mg, but with more variation than the protein values. The average ratio of protein : carbohydrate was approximately 3 : 1.

4.1.2.3. Inhibitory activity towards yeast cells and buccal epithelial cells

Ideally, it would have been desirable to test MPA for ability to inhibit the adhesion of germ tubes to buccal epithelial cells (BEC). However, it was decided in the first instance to use yeast cells rather than germ tubes because a simple method had been developed in this laboratory by McCourtie and Douglas (1981). These investigators had used this technique for demonstrating adhesion-inhibition by extracellular polymeric material (EPM) from yeast cells of *C. albicans*.

In the present experiments, 10^5 BEC were incubated with the MPA at 1 mg ml^{-1} of protein before mixing with 10^7 *C. albicans*, followed by further incubation and passage through a membrane filter with 12 µm pores. The filter was washed with PBS to remove unattached yeast cells, fixed with methanol, gram-stained and examined under bright-field microscopy at 400x. A control preparation similar to the above consisted of BEC preincubated with PBS instead of MPA. The number of adherent *C. albicans* per 100 epithelial cells was counted and the average number of yeasts per BEC determined. It is clear from Table 7 that MPA at 1 mg ml^{-1} had no detectable

Table 6. Biochemical analysis of cell wall extract of germ tubes from petri dishes

Experiment	Protein (µg/ml)	Carbohydrate (µg/ml)	Ratio [a]
1	1.68	0.39	4.3
2	1.55	0.41	3.7
3	1.68	0.16	1.5
Mean ± SEM	1.64 ± 0.04	0.32 ± 0.09	3.16 ± 0.84

[a] protein / carbohydrate

Table 7. Effect of mannoprotein adhesins (MPA) of *C. albicans* at 1 mg ml^{-1} on the adhesion of the yeast to buccal epithelial cells.

Treatment of BEC	Mean of adherent [a] yeast cells / BEC± SEM	P[b] (%)
MPA + *C. albicans*	18.3+1.5	10
PBS + *C. albicans*	18.2+1.8	10
Control (BEC only)	0.2+ 0.04	0.1

[a] Assay done in triplicate
[b] Probability values relative to adhesion of PBS + the yeast cells (control for MPA + the yeast cell)

inhibitory activity towards the adhesion of *C. albicans* to BEC. No further experiments in this area were performed.

4.2. DEMONSTRATION OF LECTIN-LIKE ADHESINS OF *C. ALBICANS*

The major focus of the studies in this thesis was to identify and quantitate the lectin-like adhesins (sugar specific proteins) both on yeast cells and on hyphal-form *C. albicans.* For this purpose synthetic glycoproteins (neoglycoproteins) consisting of sugars convalently linked to BSA which was then labelled with fluorescein isothiocyanate (FITC) were employed (Monsingy *et al.*, 1984). These neoglycoproteins were then investigated with a variety of techniques.

4.2.1. Fluorescence Microscopy

Yeast cells of *C. albicans* were obtained by growth in two media: Modified Lee Medium without amino acids (MLMwAA) and Yeast Nitrogen Base Medium with Galactose (YNBGal). The cells were harvested, washed and treated with the fucose probe (Fuc-BSA-FITC) and exanined and photographed at magnifications of 400x and 1000x, with bright field and also in blue light. The other probes, Man-BSA-FITC, Glc-BSA-FITC, Gal-BSA-FITC, Lac-BSA-FITC were also used.

The fucose probe was observed to bind to yeast-form cells which had been grown in different media. Figure 16A shows unstarved yeast-form cells grown in YNBGal. Figure 16B represents the same form cells under starved condition in MLMwAA. The photographs of the cells in the same field were taken under bright field and blue light. The small spots of the fucose probe on the cells for both conditions were observed under blue light microscopy.

Germ tube (hyphal-form cells) production with starved yeast cells in MLMwAA which had been incubated in 199 Medium for 2.5 h and 4 h at 37°C was observed after

labelling with the fucose probe, and their photographs are represented in Figure 16C and Figure 16D for 2.5 and 4 h respectively. For the same purpose, Glucose-Glycine Medium was also used to produce germ tubes during 4 h incubation at 37°C and its photograph is shown in Figure 16E.

There were four patterns of labelling with the fluorescent probe. In the micrograph Figure 16C; a comparison of the bright field and blue light photographs shows a hyphal-form cell with fluorescent label at the tip and in the region of attachment of the yeast portion; the latter remained unlabelled. The second pattern (Figure 16C; b) shows both yeast and the tip of the germ tube with the fluorescent label. In the third pattern (Figure 16C; c), the yeast portion is strongly labelled while the germ tube portion is labelled proximal to the parent cell and with a small spot of fluorescence at the tip. As regards abundance, the predominant cell type was the one represented in Figure 16C; d, e, f and showing uniform surface labelling of the germ tube portion attached to an unlabelled yeast portion of the hyphal-form cells.

The photographs of the hyphal-form cells grown for 4 h in 199 Medium showed binding of the fucose probe to both portions of the surfaces (Figure 16D; a, long arrow). Although the probe appeared on the yeast cell portion, it was mostly located on the germ tube portion. In the same photograph, the other cell which is shown with the short arrow has the probe only on the germ tube portion of the cell. But strong binding of the probe was on the adjacent point of germ tube portion with the yeast cell portion. The probe was mostly observed on the germ tube portion of the cells and in a bound patchy fashion on the tip, middle and end of the germ tube portion (Figure 16D; b, long arrow). The other hyphal cell in the same photograph contained the probe only on the tip of the germ tube portion (Figure 16D; 2, short arrow).

The hyphal-form cells grown in GG Medium for 4 h sometimes showed a patchy appearance of the probe on the germ tube portion. In this case the tips of the germ tube portions were labelled stronger than other parts of the whole cells (Figure 16E, long arrow). The other cell (short arrow) appeared to bind the probe on both

portions of the cell. There was also stronger fluorescence on the yeast cell portion (Figure 16E, short arrow).

Hyphal-form cells exposed to labelling with the mannose, the glucose, the galactose and the lactose probes did not show much fluorescence intensity on the cells. Micrographs were difficult to obtain because of the quenching problem which was associated with the exposure time.

4.2.2. Image Analysis

Hyphal-form *C. albicans* labelled with Fuc-BSA-FITC was observed by computer-assisted video-image analysis. The hyphal-form cells were produced in 199 Medium by 2 h incubation at 37°C. The binding procedure of Fuc-BSA-FITC to the cells was the same as for conventional fluorescence microscopy. The other probes, Man-BSA-FITC, Glc-BSA-FITC, Gal-BSA-FITC, Lac-BSA-FITC were also employed.

With each probe, four types of images were obtained, as illustrated in the subsections of a hyphal *C. albicans* labelled with the fucose probe, in alphabetical order: a = bright-field image; b = contour plot; c = blue light image; d = perspective plot of blue light image.

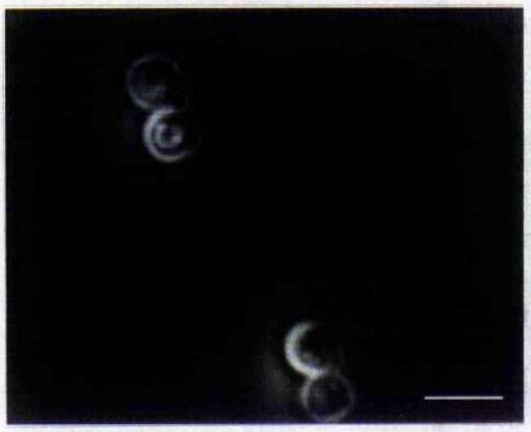
The Figure 17A shows both the actual records from the instrument (top, a and c) and the interpretations (below, b and c). It is clear that the fucose probe had labelled both the tip of the germ tube portion and a restricted area on the yeast cell. This type of image was typical of those seen with the fucose probe (Appendix 5.1; a, b, c, d). However, the fucose probe bound on the yeast cell portion (Appendix 5.1; e) or both portions of the cell (Appendix 5.1; f).

The mannose probe gave a similar but less distinct picture (Figure 17B). Although the glucose and galactose probe were detectable with image analysis, their fluorescence were mostly on the germ tube portions (Figure 17C and D). The hyphal-form cells did not show any fluorescence when it was labelled with the lactose probe (Figure 17E).

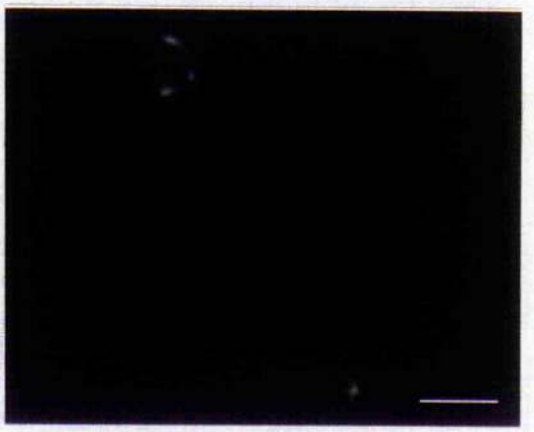
Figure 16. Fluorescence microscopy of *C. albicans* with the fucose probe. Cells were grown in various media to retain yeast cells (A, B) or to induce germ tube production (C, D, E) and examined by brigth field or in blue light. Bars represent 5 μm

A. The yeast-form cells grown in YNBGal (unstarved) 1000 x

Bright field

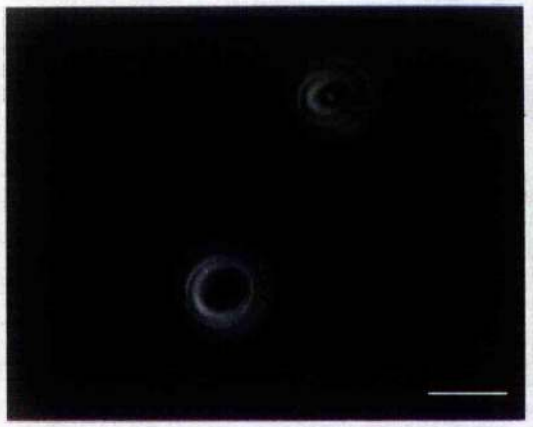


Blue light



B. The yeast-form cells grown in MLMwAA (starved) 1000 x

Bright field

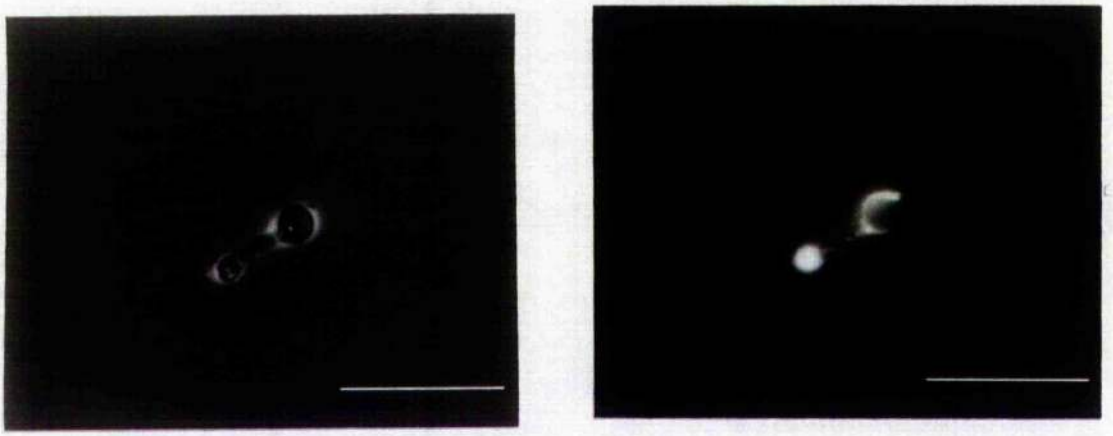


Blue light

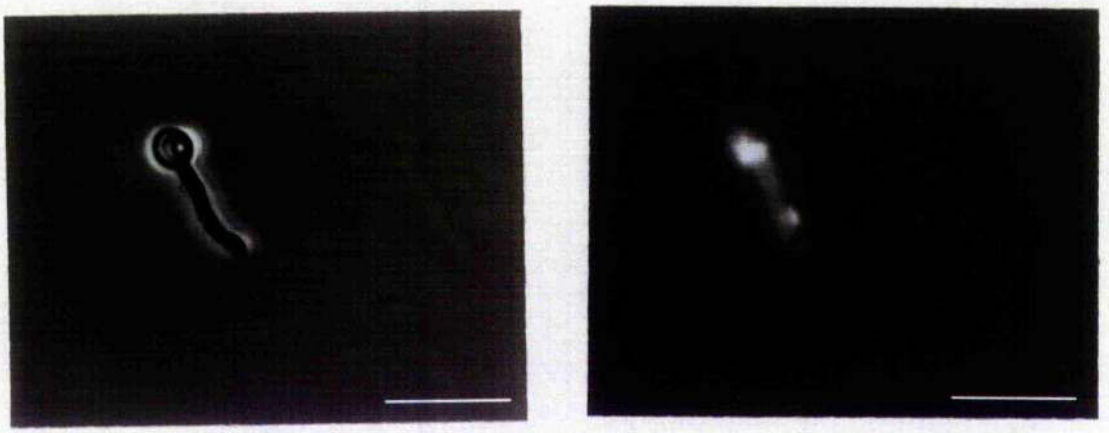
The hyphal-form cells illustrated according to their labelling patterns with the fucose probe as a, b, c, d, e, f. Each cell was photographed with Bright field and Blue light.

C. The hyphal-form cells grown in 199 Medium for 2.5 h at 37°C 1000 x. Bars represent 10 μm

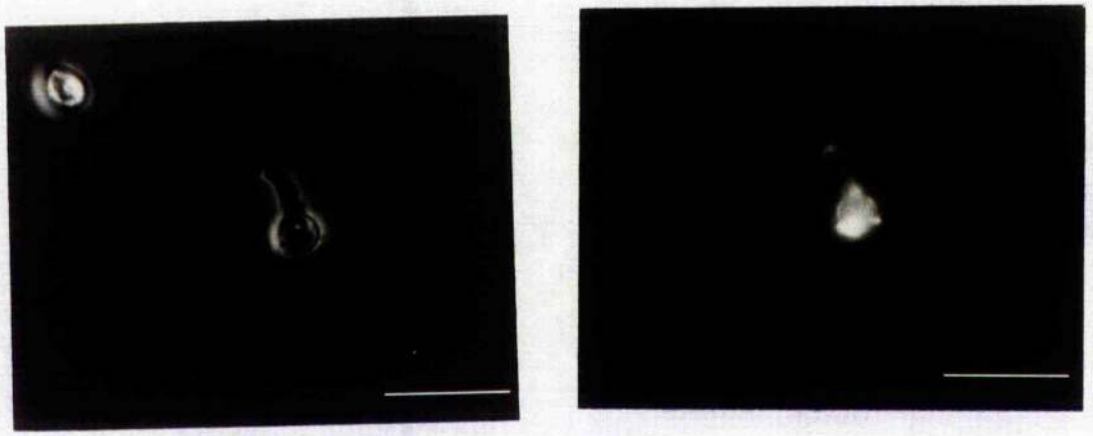
a. Bright field Blue light



b. Bright field Blue light

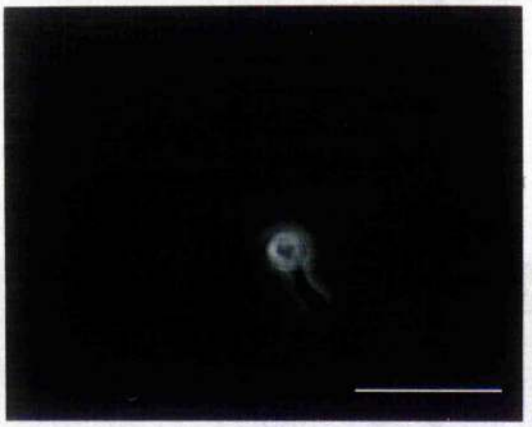
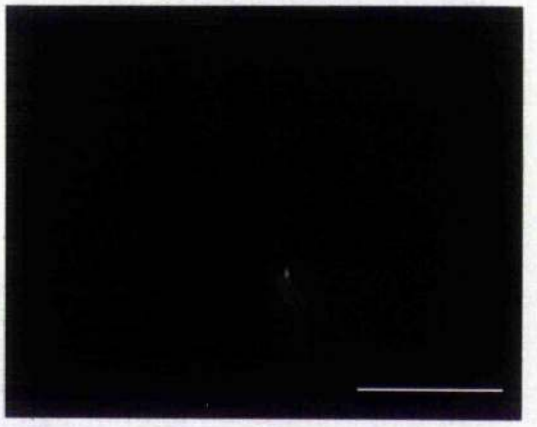


c. Bright field Blue light

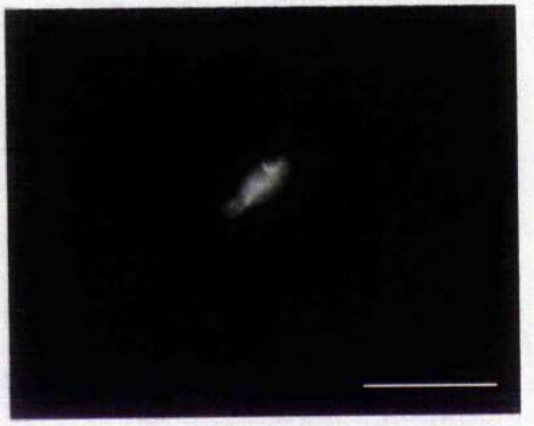


continued....

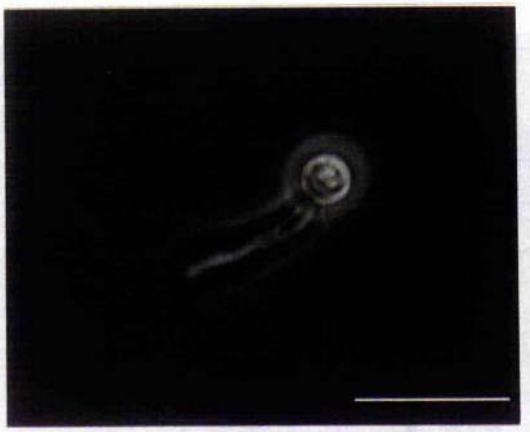
d. Bright field Blue light

e. Bright field Blue light

f. Bright field Blue light

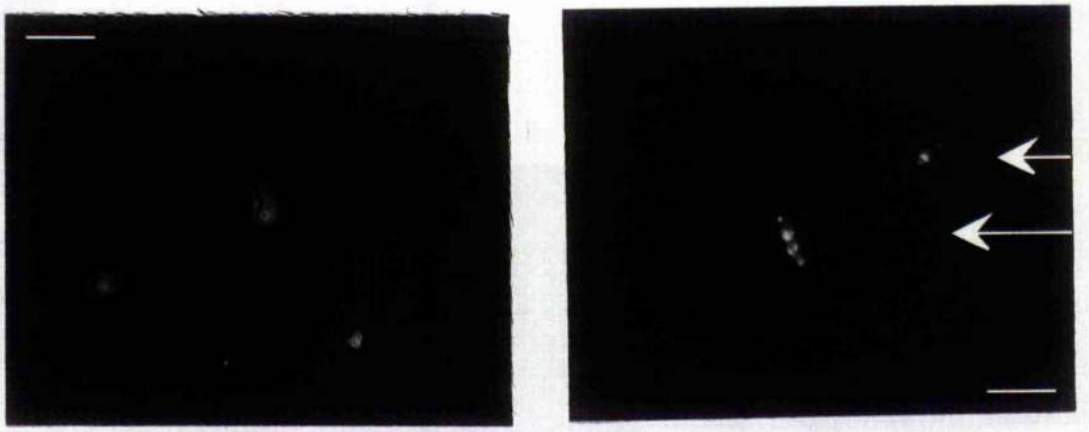



The hyphal-form cells illustrated according to their labelling patterns with the fucose probe as a, b. Each cell was photographed with Bright field and Blue light. Arrows indicate the cells labelled with the fucose probe.

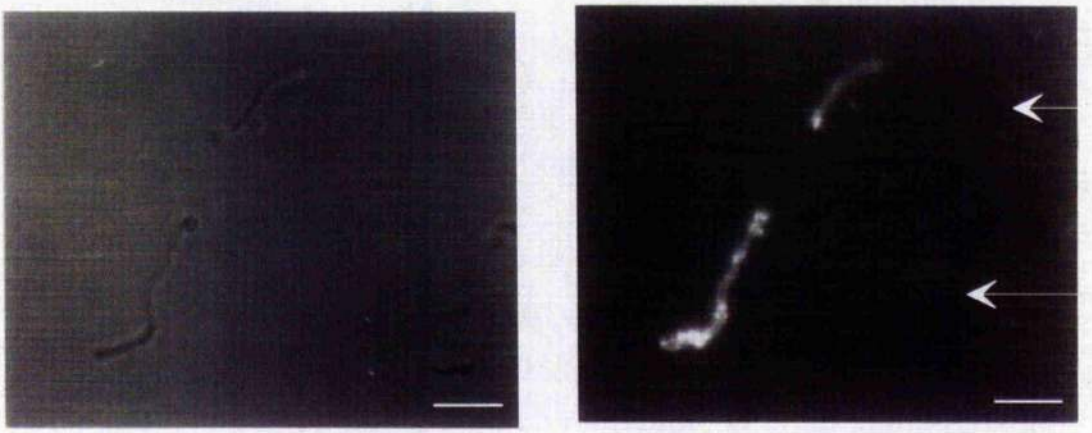
Each cell was photographed with Bright field and Blue light. Arrows indicate the cells labelled with the fucose probe.

D. The hyphal-form cells grown in 199 Medium for 4h at 37°C 400 x. Bars represent 10 μm

a. Bright field Blue light

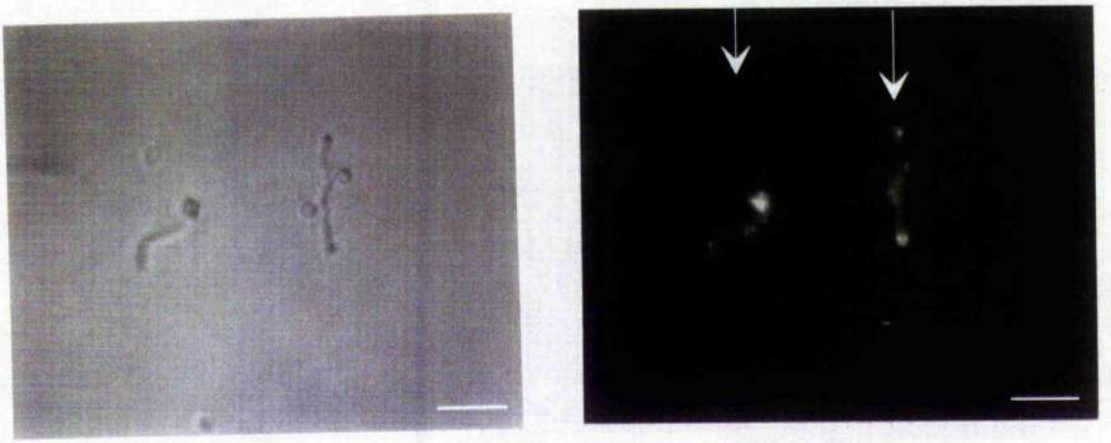


b. Bright field Blue light



E. The hyphal-form cells grown in 199 Medium for 4 h at 37°C 400 x. Bar represents 10 μm.

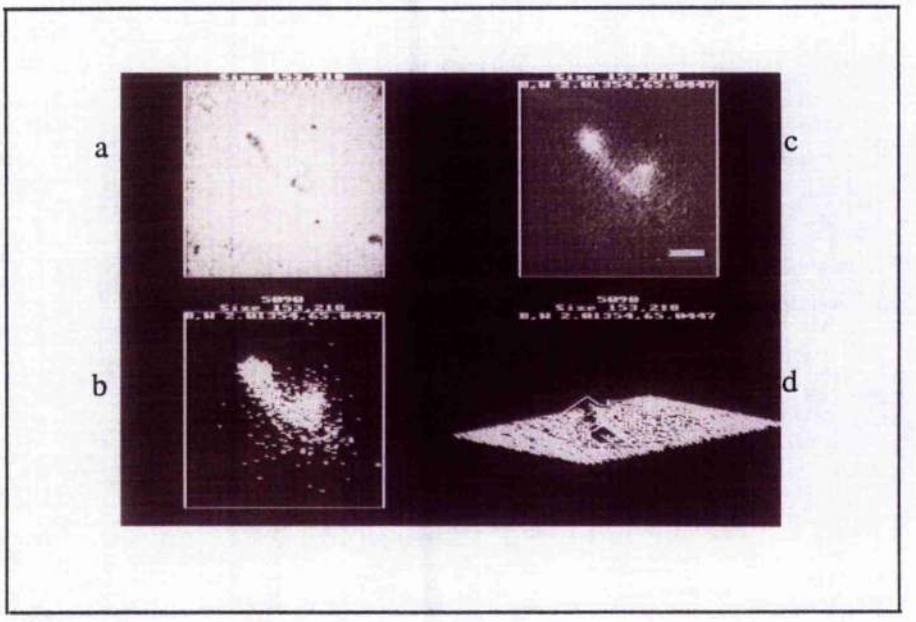
Bright field Blue light

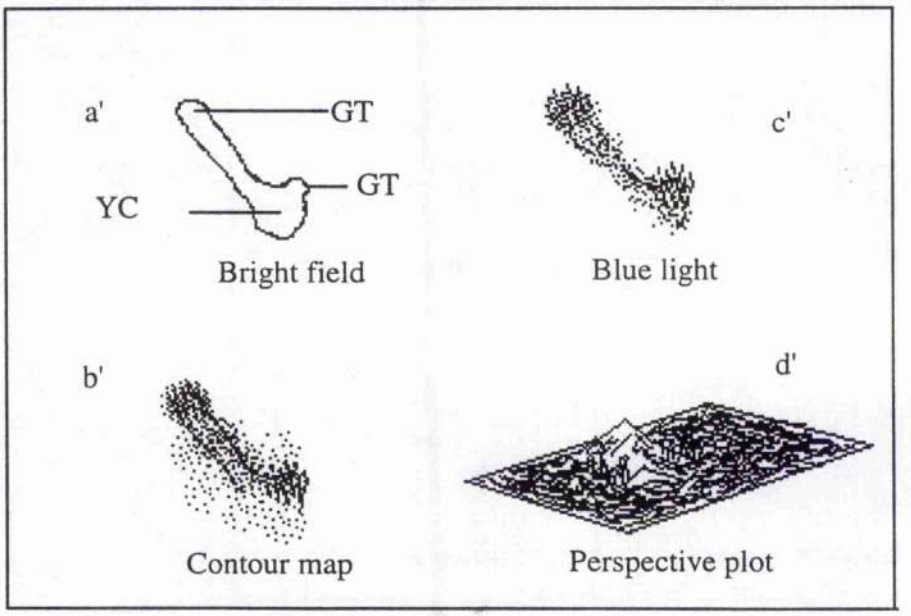


These probes were Fucose (A), Mannose (B), Glucose (C), Galactose (D), Lactose (E). Printer papers illustrated a = bright-field image; b = contour plot; c = blue light image; d = perspective plot of blue light image. The interpretation of printer paper shows same images as a', b', c', d'.

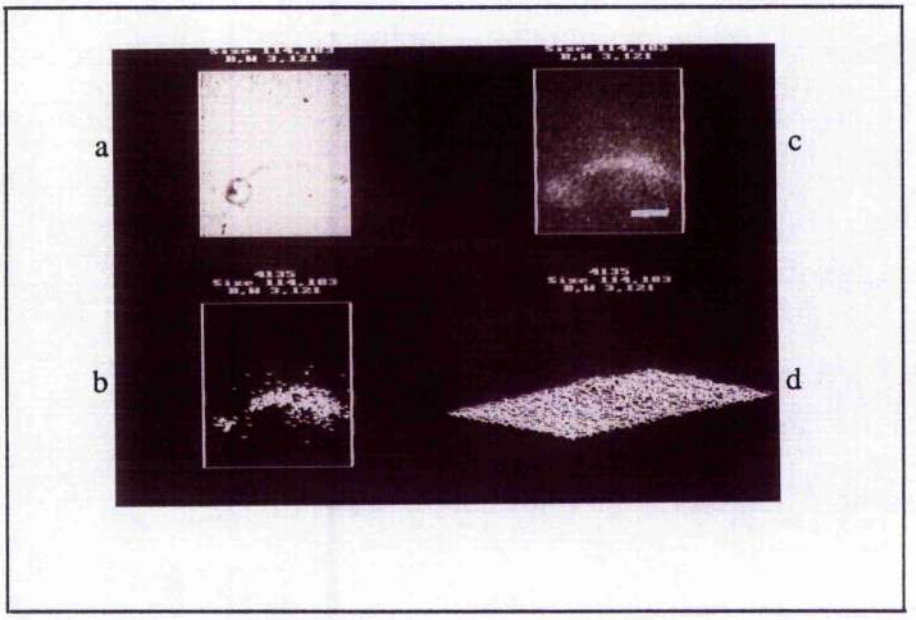
Figure 17. Image analysis of *C. albicans* by fluorescence microscopy : Printer papers of computer output of the hyphal-form cells labelled with individual probes (upper composite) and interpretation (lower composite) 1000 x. Bar represent 2.5 μm

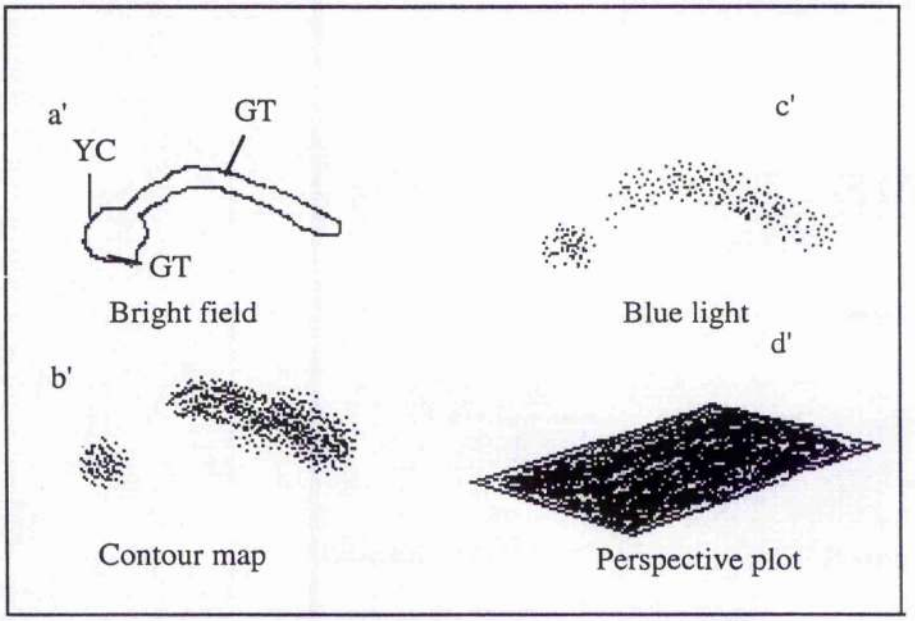
A. Fucose probe



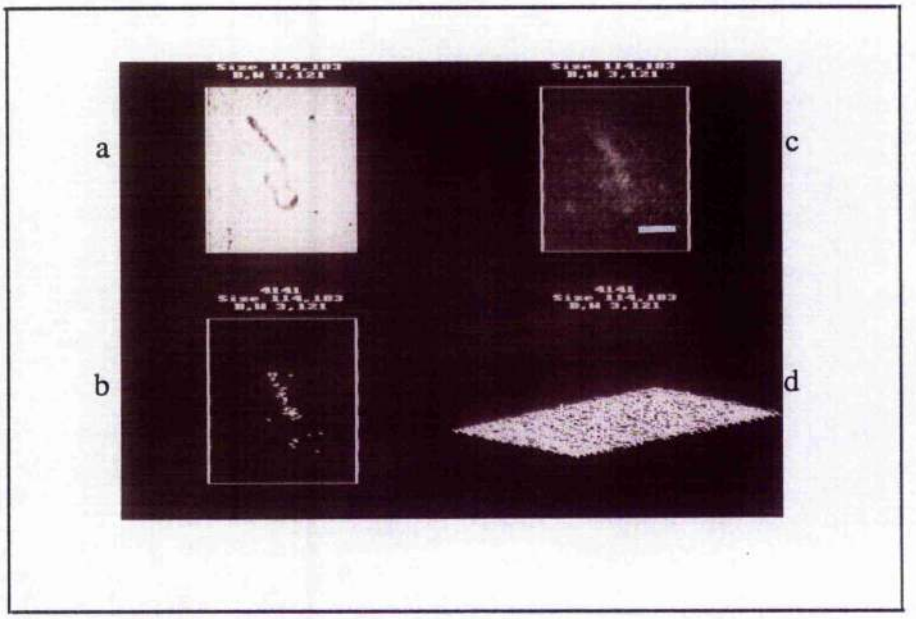


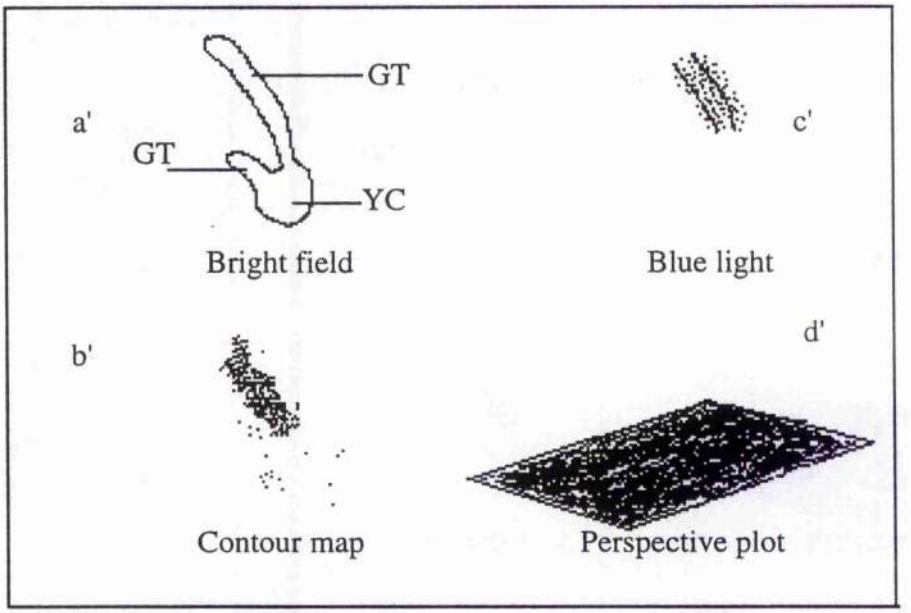
B. Mannose probe



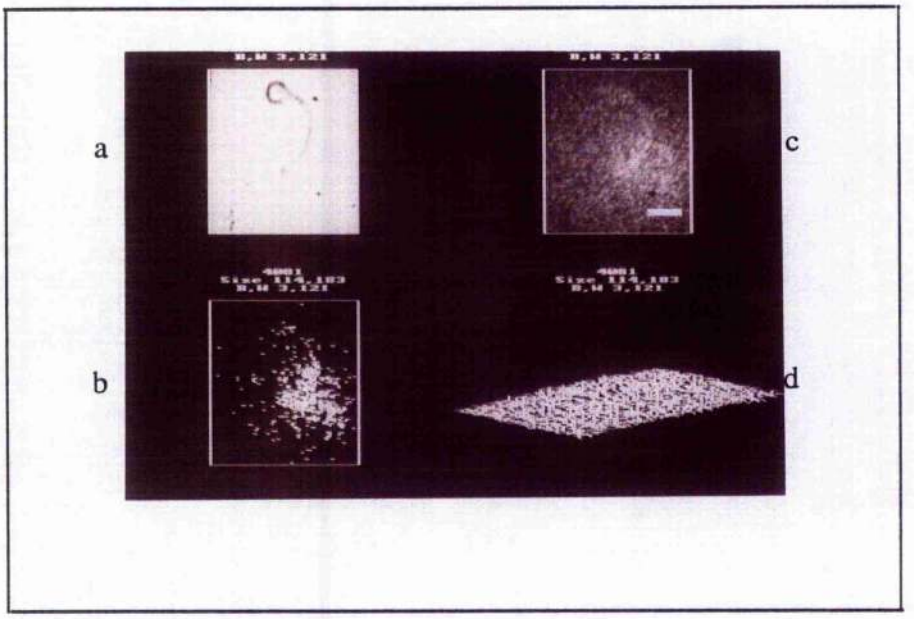


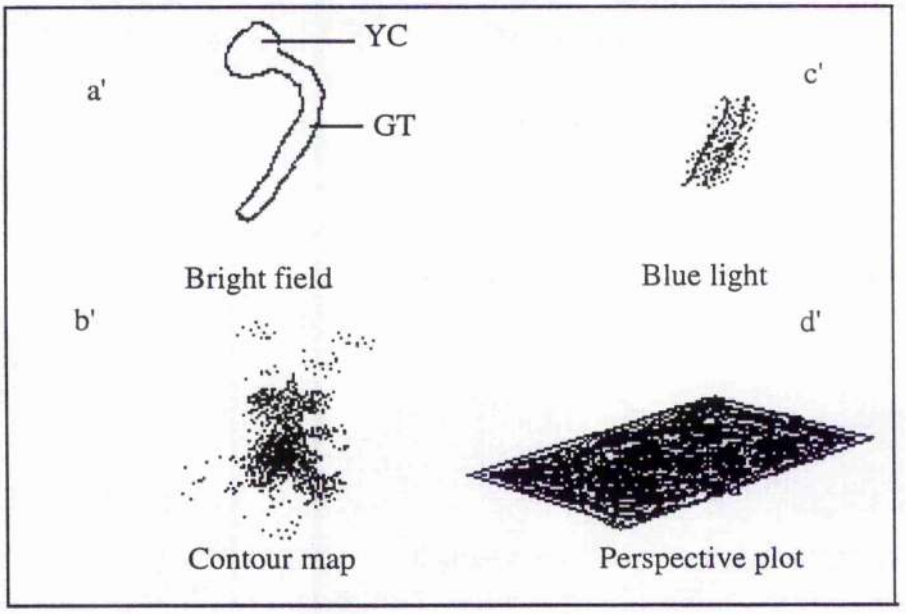
C. Glucose probe



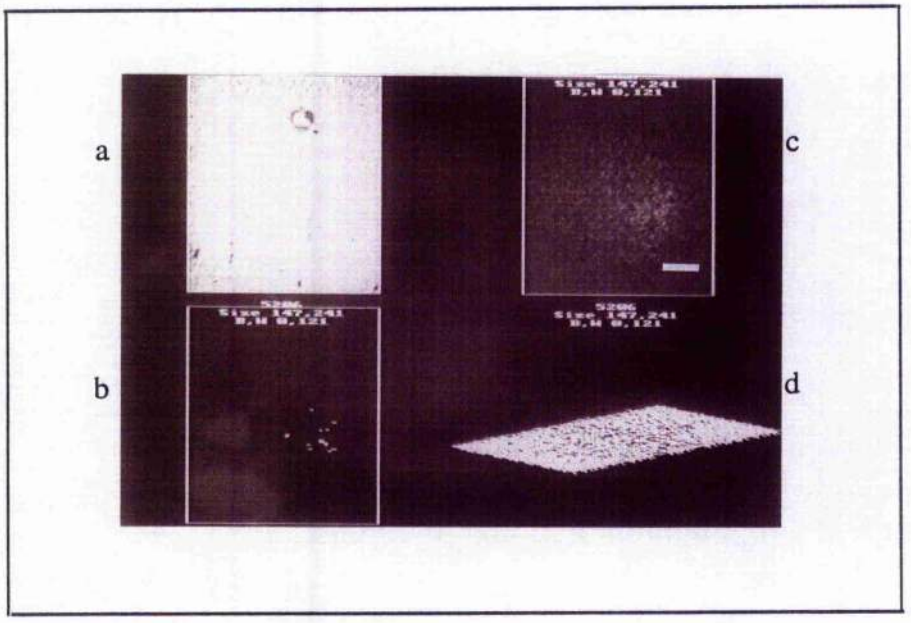


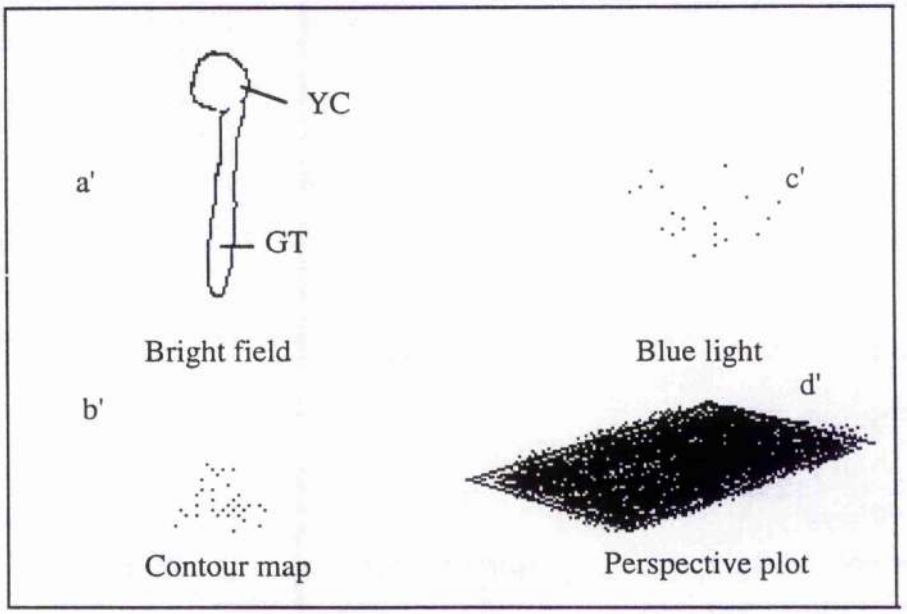
D. Galactose probe





E. Lactose probe





4.3. QUANTITATION OF LECTIN-LIKE ADHESINS OF *C. ALBICANS*

4.3.1. Spectrofluorimetry with Yeast-Form Cells

The specificity of binding of neoglycoprotein probes labelled with fluorescein was studied using both the yeast and the hyphal-forms of *C. albicans*, the former being examined first. The neoglycoprotein Fuc-BSA-FITC consisted of fucose coupled to BSA and labelled with fluorescein, in the approximate molecular ratio 16 : 1: 2.3. The probe (200 μg ml^{-1}) was dissolved in 0.1 M citrate buffer at pH 5.0 supplemented with 1% (w/v) BSA to prevent non-specific binding. It was added to the yeast cells ($2x10^8$ cells ml^{-1}), which had been grown on YNBGal, and incubated for 1 h at 37°C to allow labelling. This incubation was done in the dark as a precaution against possible instability of fluorescein. After incubation, the labelled yeast cells were harvested by centrifugation and the supernate which contained the unbound reagent was discarded. The pellet of cells was then washed twice in PBS pH 7.4, after which the FITC bound to the yeast cells was released by pronase (0.1 mg ml^{-1}) treatment at pH 7.4 for 2 h at 37°C. The released FITC was quantitated by spectrofluorimetry at 520 nm, with an excitation wavelength of 495 nm, and the fluorescence was recorded in arbitrary units on a scale of zero to 30. The background fluorescence of the probe was substracted. This general procedure was used to investigate the variables detailed below.

Note : All of the figures in section 4.3.1 are presented together after page 110.

4.3.1.1. Effect of pH

In a preliminary experiment, the binding of Fuc-BSA-FITC to the yeast cells was studied over the pH range from 3.0 to 8.0, using 0.1 M citrate buffers for pH 3.0, 4.0 and 5.0, and 0.1 M phosphate buffers for pH 6.0 - 8.0. All of these buffers contained 1% (w/v) BSA to prevent non-specific binding. Figure 18 shows that binding of Fuc-BSA-FITC to the yeast cell was strongly influenced by pH, with maximum binding

taking place around pH 5.0 and being much reduced at pH 4.0 and 6.0. It was not possible to obtain proper results with the samples at pH 3.0 because of precipitation of the probe.

4.3.1.2. Effect of incubation time

Having established pH 5.0 in citrate buffer as suitable for these binding studies, the effect of incubation time with the fucose probe was explored. Exposures of 15, 30, 60, 90 and 120 min were selected, and after each exposure the sample was put on ice to stop the reaction. After completing the 120 min incubation period, all the samples were washed twice and treated with pronase as before. The results of the single experiment show (Figure 19) that time was an influential variable. Thus the binding at 15 min was much less than 60 min after which the process levelled off. In subsequent experiments an incubation time of 60 min was routinely used.

4.3.1.3. Saturation curve

To demonstrate the saturability of specific binding, the fluorescein-labelled neoglycoprotein (Fuc-BSA-FITC) was used in concentrations ranging from 3.1 up to 400 μg per ml. Results with a 60 min exposure time at pH 5.0 are presented in Figure 20. It is clear that with 10^8 yeast cells, the fucose binding sites become saturated at a probe concentration of 100 μg ml^{-1}.

4.3.2. Spectrofluorimetry with Hyphal-Form Cells

4.3.2.1. Effect of concentration of pronase

Because of the light-absorbing effect of cell opacity, it was necessary to solubilize the probe before spectrofluorimetry. Pronase B (45 000 PUK g^{-1}) at 0.1 mg ml^{-1} in PBS pH 7.4 was used remove the probe from the cell wall by Depierreux *et al.*, (1991). In the present experiments, the hyphal-form cells were labelled with 200 μg fucose probe ml^{-1} (as final concentration) in 0.1 M Citrate buffer at pH 5.0 and 0.1 M phosphate

buffer at pH 7.2. After washing the cells with PBS at pH 7.4, pronase E (4000 unit g^{-1}) was added to the cell pellets at concentrations of 0.1, 0.5, 1.0, mg ml^{-1}. After pronase digestion, the supernates were examined by spectrofluorimetry and the fluorescence recorded in arbitrary units (SFAU : spectrofluorimetry arbitrary units) on a scale from zero to a value in the range 0 - 100. Figure 21 (Note : All of the figures in section 4.3.2 are presented together after page 111) shows the fluorescence intensity of the released probe from hyphal-form *C. albicans* with different concentrations of pronase. Between 1.0 and 0.1 mg ml^{-1} the fluorescence intensity did not change substantially, but was higher in citrate buffer pH 5.0 than in phosphate buffer. After pronase treatment even at the lowest level (0.1 mg ml^{-1}), the cells observed under the fluorescence microscopy showed no probe remaining on the cell wall.

4.3.2.2. Effect of cell concentration

To investigate the specificity of binding of various neoglycoproteins (labelled with FITC) to the hyphal-form, it was first necessary to produce a standardized suspension of hyphal-form *C. albicans* . For this purpose, the hyphal-form cells at concentrations between 10^4 and 10^8 ml^{-1} were reacted with 100 µg ml^{-1} of the fucose probe (final concentration) for 1 h at 37°C. They were then washed and digested with pronase to remove FITC from the bound neoglycoprotein on the cells. The supernates from the pronase digestion were examined by spectrofluorimetry and the fluorescence recorded in arbitrary units on a scale from zero to a value in the range 0 - 100 which varied from day to day. The results of three independent experiments each with duplicate readings without subtracted background fluorescence (Figure 22) show that the fluorescence intensity of hyphal-form *C. albicans* at concentrations between 10^4 and 10^6 cells ml^{-1} was low, whereas 10^7 and 10^8 cells ml^{-1} gave appreciable signals in a dose-related fashion. It may be noted that all the values in Figure 22 had background fluorescence values which were not subtracted. For subsequent labelling experiments, 10^7 cells ml^{-1} were used as the final concentration of the hyphal-form cells.

4.3.2.3. Effect of pH

Using hyphal-form *C. albicans* at $2x10^7$ cells ml^{-1}, the effect of pH on the binding of five different neoglycoproteins labelled with FITC was investigated. In addition to the fucose probe described above, similar probes with mannose, glucose, galactose, and lactose were used. The experiment with the fucose probe was repeated twice, with duplicate observation for each. Figure 23A shows that only the fucose probe (Fuc-BSA-FITC) gave appreciable binding, with a maximum at pH 5.0. Binding of the other probes to the cells was done twice on the same day. The mannose probe, although giving only very low binding, also showed a poorly-defined maximum at pH 5.0 (Figure 23B). The glucose, galactose and lactose probes showed no significant pH effects in the low levels of binding exhibited (Figure 23C, D, E).

Experiments were also performed in the pH range 3.0 - 4.0, within which a granular precipitate was frequently, but not invariably, observed. Pronase treatment of these granular precipitates released soluble fluorescence of intensity about 5 times higher than the peak of fluorescence in the specific binding with the fucose probe at pH 5.0. An example of this pH 3.5 'binding' with the lactose probe, which is regarded as non-specific, is shown in Appendix 4.2.

Based on these observations, 0.1 M citrate buffer at pH 5.0 was used in all subsequent binding experiments.

4.3.2.4. Effect of incubation time

To determine the most suitable incubation time for binding of Fuc-BSA-FITC to hyphal-form *C. albicans*, experiments were done by the same method as previously used for yeast-form cells. Figure 24 shows two separate experiments, each with duplicated observations. It is clear that the process of binding of the labelled fucose probe was a time-dependent reaction requiring between 30 and 60 min, after which a plateau was reached.

Based on these results, a standard incubation time of 60 min was adopted in subsequent probe-binding studies. This is the same as had previously been established for yeast-form cells.

4.3.2.5. Saturation curve

As reported above with yeast cells, the hyphal-form cells were similarly tested to demonstrate the saturability of specific binding of the fluorescein-labelled neoglycoprotein. For this purpose Fuc-BSA-FITC was used in concentrations ranging from 3.1 up to 400 μg per ml in two days, the experiment being repeated twice on each day. Results with a 60 min exposure time at pH 5.0 are presented in Figure 25. It is clear that with 10^7 hyphal-form cells, the fucose binding sites were starting to become saturated at a probe concentration of 100 μg ml^{-1}. This was similar to the results with yeast cell without germ tube production (Figure 20).

Afterwards, for reasons of economy, most of the experiments were done with the probe at 100 μg ml^{-1}.

4.3.2.6. Standard curves of different probes

Five different probes, consisting of neoglycoproteins labelled with FITC, were dissolved and serially diluted over the range 0.6 to 40 μg ml^{-1} in 0.1 M citrate buffer pH 5.0 and assayed in the spectrofluorimeter. Dose-response relationships which were approximately linear (Figure 26) were obtained with each probe but with a four-fold range of slopes. Repetitions of the experiments on other days (Appendix 4.3) gave similar results, with the lactose and galactose probes showing strongest fluorescence and the fucose probe being consistently the weakest. Glucose and mannose probes were intermediate.

4.3.2.7. Standard curve of the fucose probe before and after treatment with pronase

An experiment was done to compare phosphate pH 7.2 and citrate buffers pH 5.0 with and without the pronase treatment of the fucose probe. For this purpose, pronase treatment was done a) in a 1 : 10 mixture of 0.1 M pH 5.0 citrate and pH 7.4 PBS, and b) in a 1 : 10 mixture of 0.1 M pH 7.2 phosphate and pH 7.4 PBS. containing the probes. After 2 h incubation at 37°C, the fluorescence intensity of the probe was seen to increase in samples which contained pronase. Figure 27 shows that the strongest fluorescence was obtained when the probe was digested under conditions 'b' while the probe without pronase treatment gave very low fluorescence irrespective of pH. Treatment under conditions 'a' was generally similar to 'b' except at the highest dose of probe.

4.3.2.8. Effect of different probes

Previous experiments showed that the hyphal-form cells possessed fucose-specific lectin-like adhesin on their surfaces. The cells were also exposed to labelling with the other probes, the mannose, the glucose, the galactose and the lactose in the citrate buffer pH 5.0. This experiment was repeated five times on three days. The background values of neoglycoproteins were not subtracted in this experiment. As shown in Figure 28, the binding of the fucose probe was higher than the mannose. Binding capacity of the glucose probe seemed to be less than the mannose probe. The galactose and the lactose probe were not able to bind to the hyphal-form cells and gave nearly negative results.

Based on the probe containing 25 molecules of fucose per BSA molecule, and making some simplifying assumptions, it may be calculated that each hyphal-form cell of *C. albicans* grown in the199 Medium, possessed about $2x10^7$ fucose probe binding sites. Details of the calculations are set out in Appendix 4.1.

4.3.2.9. Germ tubes production under different growth conditions

Additional comparisons between hyphal and yeast-form cells for binding of the fucose probe were made with *C. albicans* . Three different condition were explored. In the first, starved cells were inoculated into 199 Medium at pH 6.7 and incubated at 22°C; in the second and third conditions a temperature of 37°C was used with the 199 Medium at pH 6.7 and pH 4.0. Incubation times ranged from 0 to 5 h. Control, starved cells in 199 Medium, were kept on ice. Two types of observation were made: microscopical observations of germ-tube production (Figure 29B) and budding, and of binding of Fuc-BSA-FITC followed by fluorescence measurements (Figure 29A). In the 199 Medium at pH 6.7 at 37°C, the first germ tubes appeared after 1 h min and extended in length during the subsequent incubation. In contrast, the other two incubation conditions gave no significant germ-tube production during the 5 h exposure. There was however budding of yeast cells at pH 6.7 and 22°C, and also at pH 4.0 and 37°C. This type of growth continued up to 5 h when the experiment was stopped.

The above results of germ tube production were closely mirrored by the binding results with Fuc-BSA-FITC. Thus only the pH 6.7, 37°C conditions gave structures that bound the fluorescence probe. Moreover, the increased binding beyond two hours corresponds to the extension in length of the germ tubes which was seen microscopically.

4.3.2.10. Effect of Ca^{++} and Mg^{++}

The effect of 2 mM Ca^{++} and Mg^{++} on binding of the fucose probe to hyphal-form *C albicans* was investigated. This experiment was done in 0.1 M citrate buffer without Ca^{++} and Mg^{++}, and acetate buffer with and without Ca^{++} and Mg^{++} at pH 5.0; 0.1 M phosphate buffer without Ca^{++} and Mg^{++} and 0.1 M tris-HCl buffer with and without Ca^{++} and Mg^{++} at pH 7.2. The experiment was repeated three times and each of them was duplicated. The results (Figure 30) showed that binding of the probe to the hyphal-form cells in buffers at pH 5.0 was better than in buffers at pH 7.2. Addition of Ca^{++} and Mg^{++} to the buffers did not affect binding of the fucose probe. This experiment also

Figure 18. Spectrofluorimetry of *C. albicans* : Effect of pH on binding of the fucose probe by the yeast-form cells

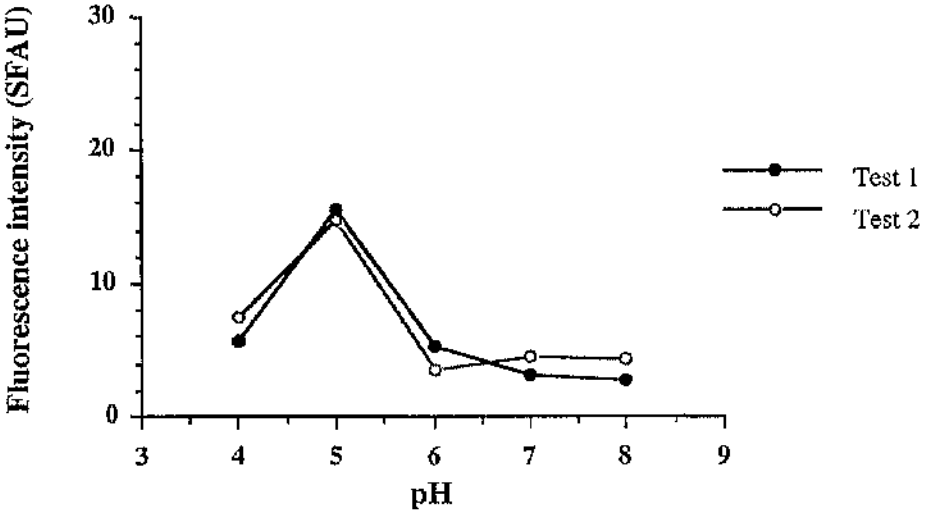


Figure 19. Spectrofluorimetry of *C. albicans* : Effect of incubation time on binding of the fucose probe by the yeast-form cells

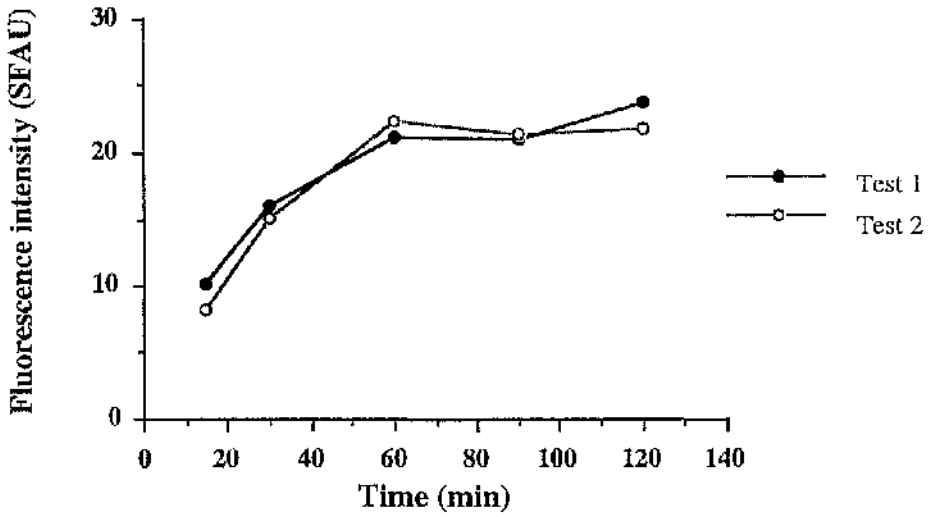


Figure 20. Spectrofluorimetry of *C. albicans* : Saturation curve of binding of the fucose probe by the yeast form cells

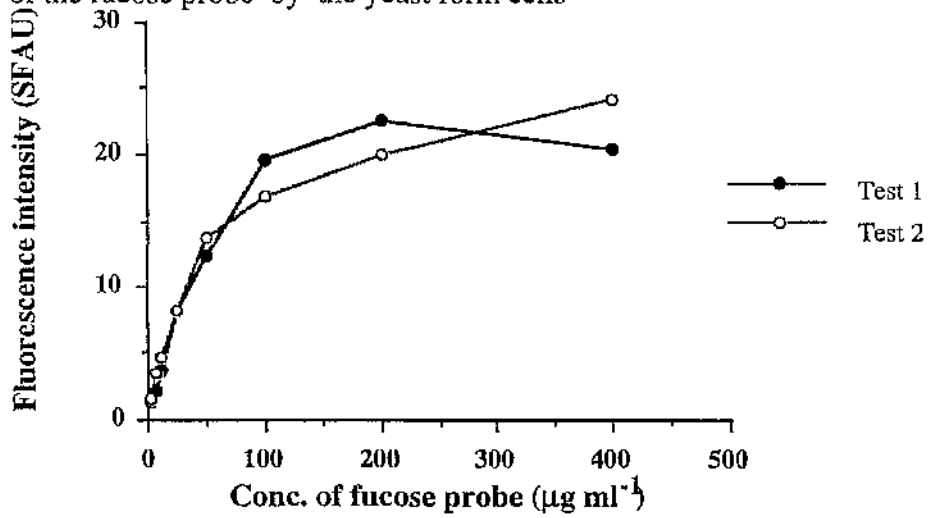


Figure 21. Spectrofluorimetry of *C. albicans*. : Effect of different pronase concentrations to remove the fucose probe from the hyphal-form cell wall

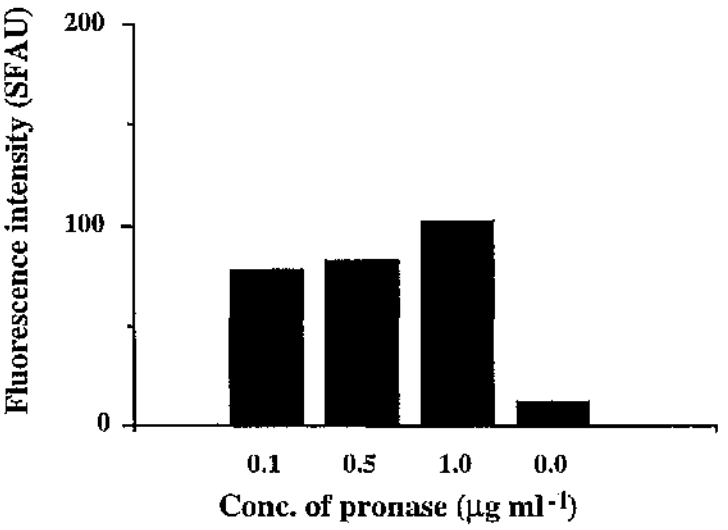


Figure 22. Spectrofluorimetry of *C. albicans* : Effect of concentration of the hyphal-form cells on binding of the fucose probe. Vertical lines represent SEM

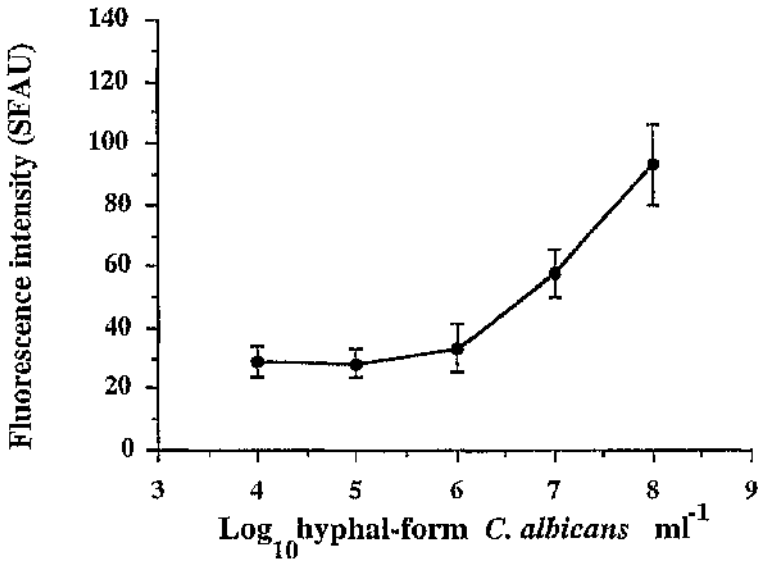
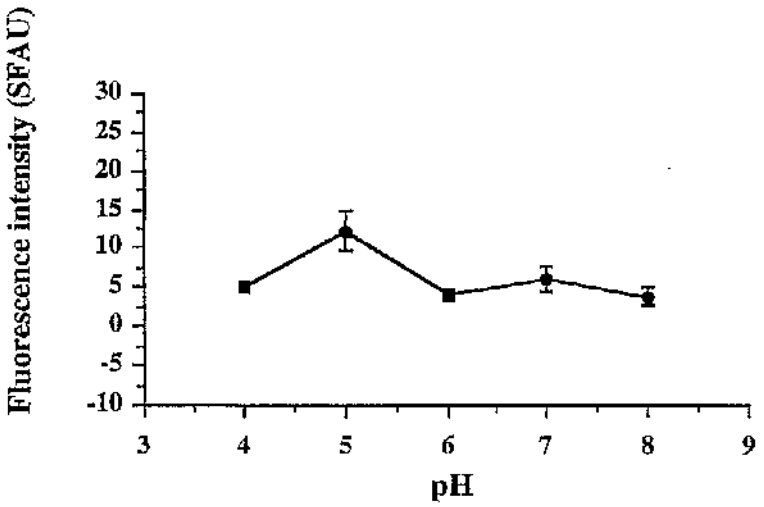
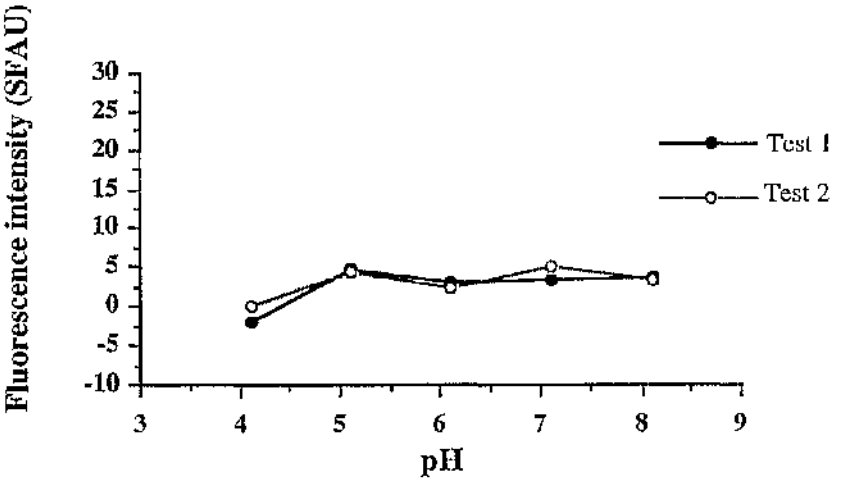


Figure 23. Spectrofluorimetry of *C. albicans* : Effect of pH on binding of five probes by the hyphal-form cells

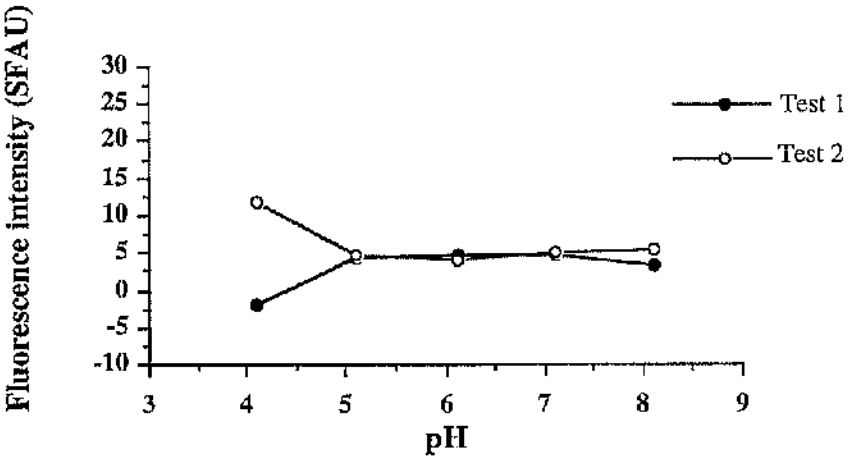
A. Fucose probe. Vertical lines represent SEM



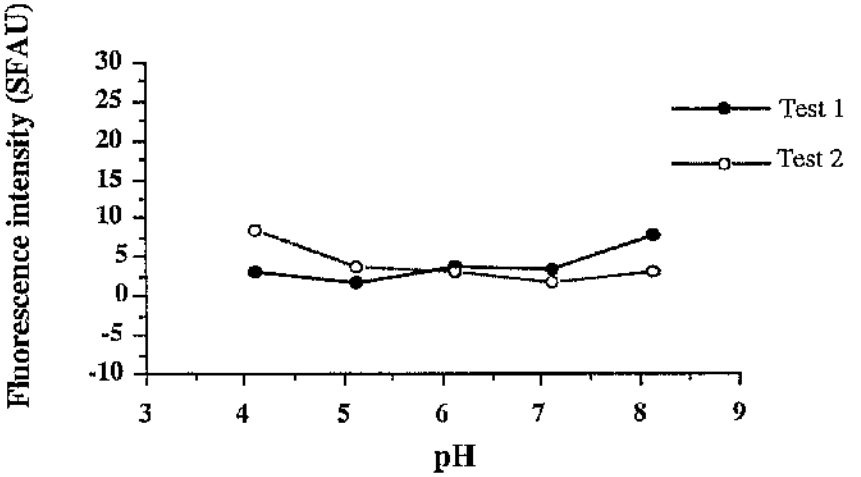
B. Mannose probe



C. Glucose probe



D. Galactose probe



E. Lactose probe

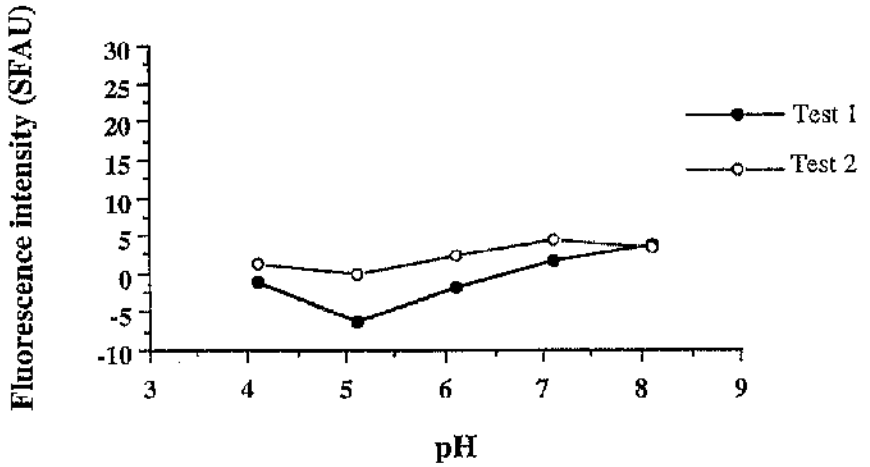


Figure 24. Spectrofluorimetry of *C. albicans* : Effect of incubation time on the binding of the fucose probe by the hyphal-form cells. Vertical lines represent SEM

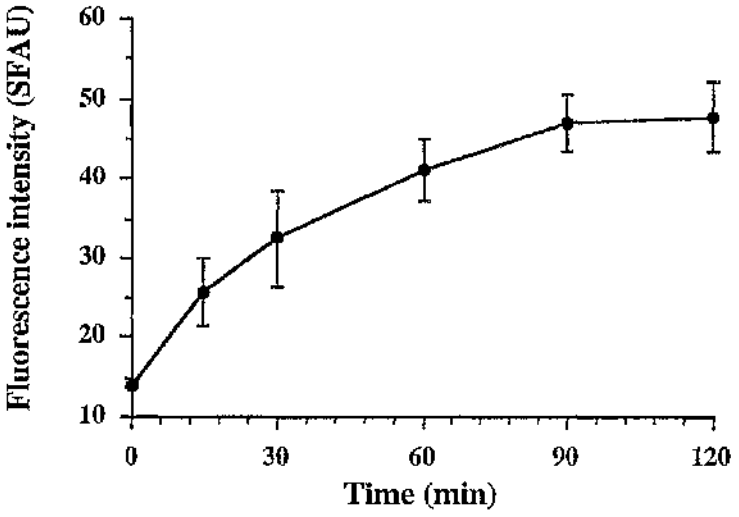


Figure 25. Spectrofluorimetry of *C. albicans* : Saturation curve for binding of the fucose probe by the hyphal-form cells. Vertical lines represent SEM

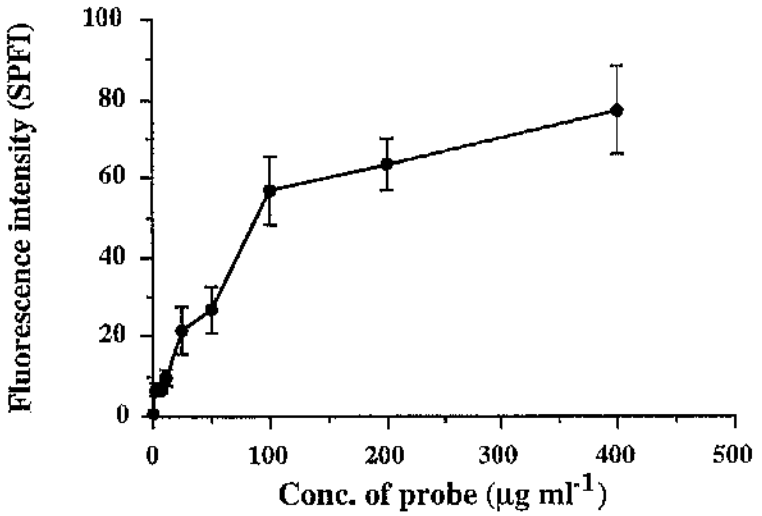


Figure 26. Spectrofluorimetry of various probes: Standard curves at pH 5.0

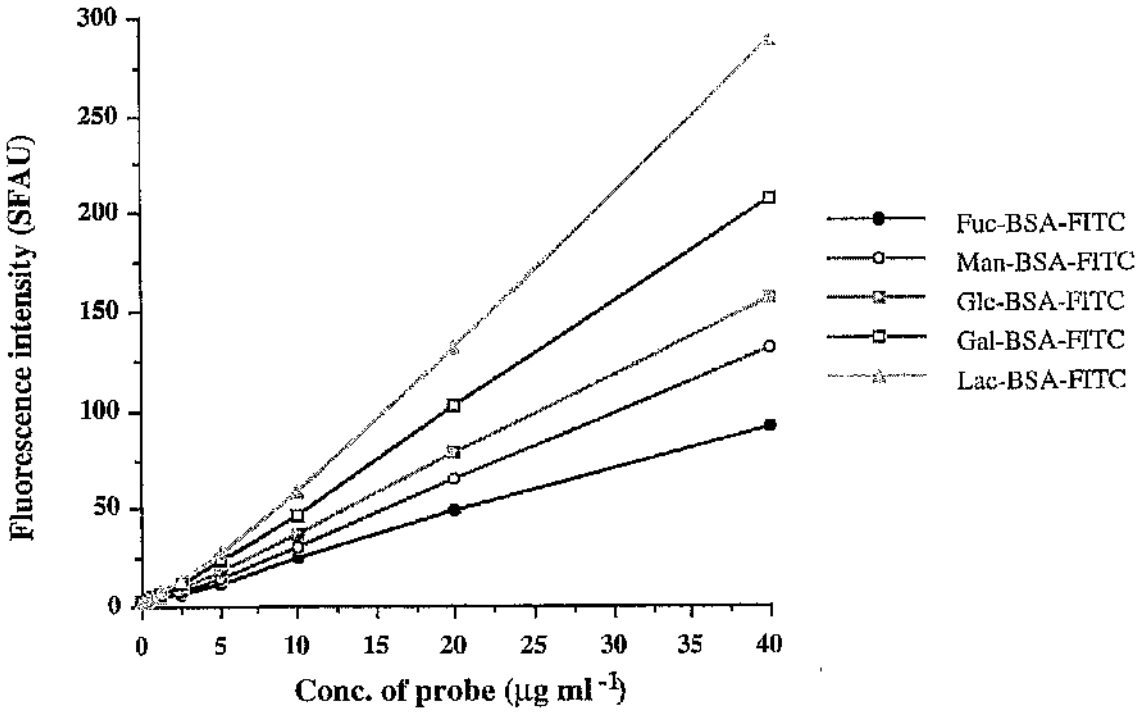


Figure 27. Spectrofluorimetry of the fucose probe: Effect of pronase treatment of the probe itself at different pH

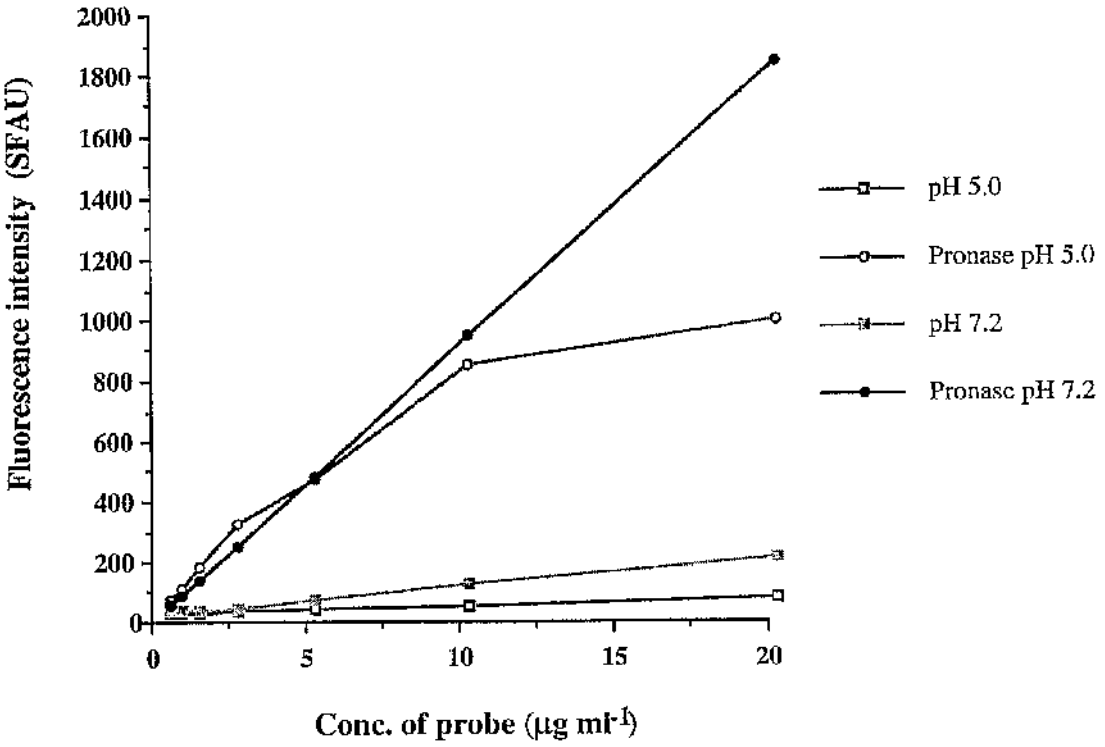


Figure 28. Spectrofluorimetry of *C. albicans* : Comparison of binding of five probes by the hyphal-form cells

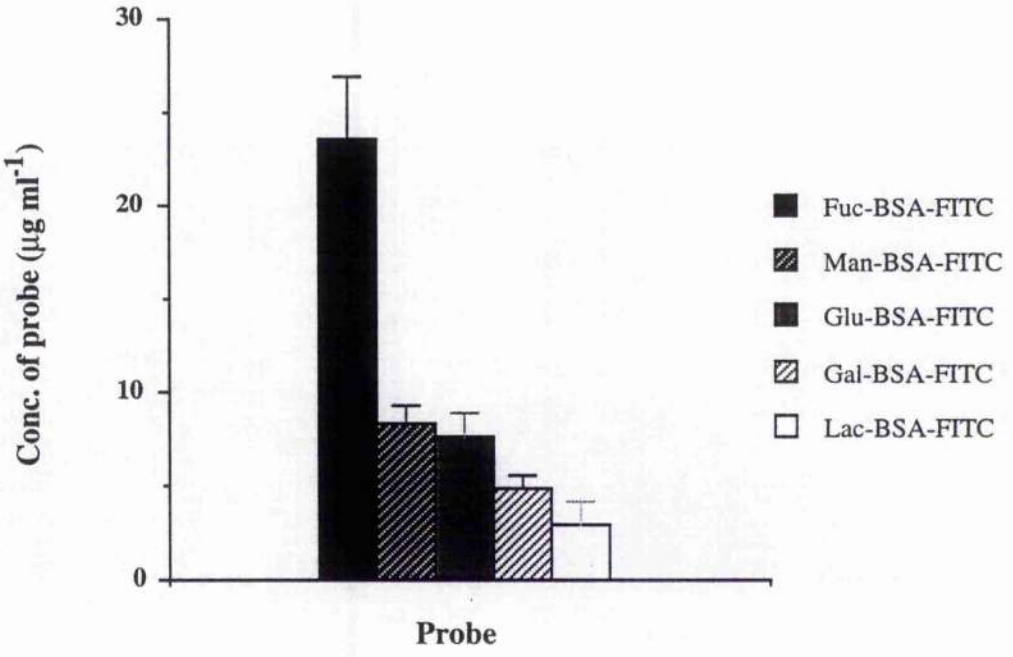


Figure 29A. Spectrofluorimetry of *C. albicans* : Effect of growth in 199 Medium at different pHs and temperatures on binding of the fucose probe by the cells during germ-tube production

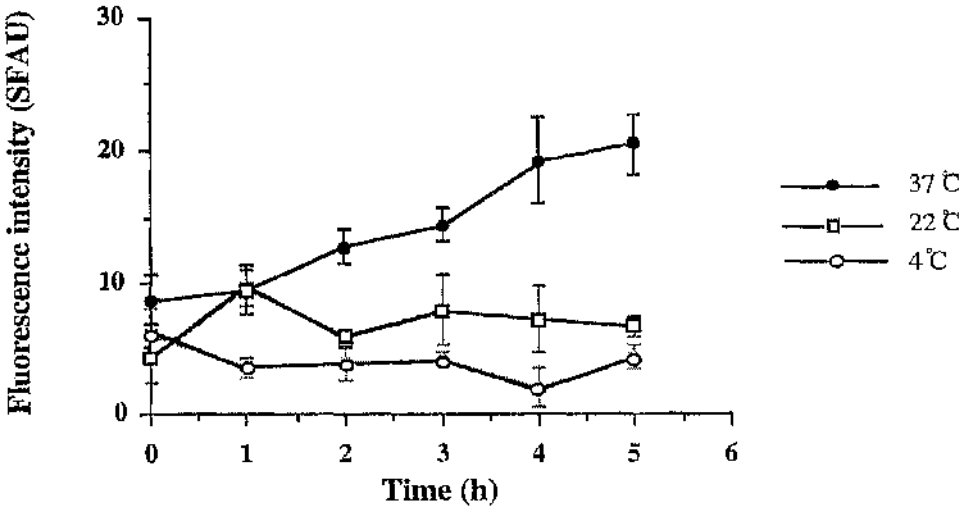


Figure 29B. Light microscopy of*C. albicans* : Effect of growth in 199 Medium at pH 6.7 and 37°C to produce germ tube. The error bars for SEM in most instances do not extend beyond the points

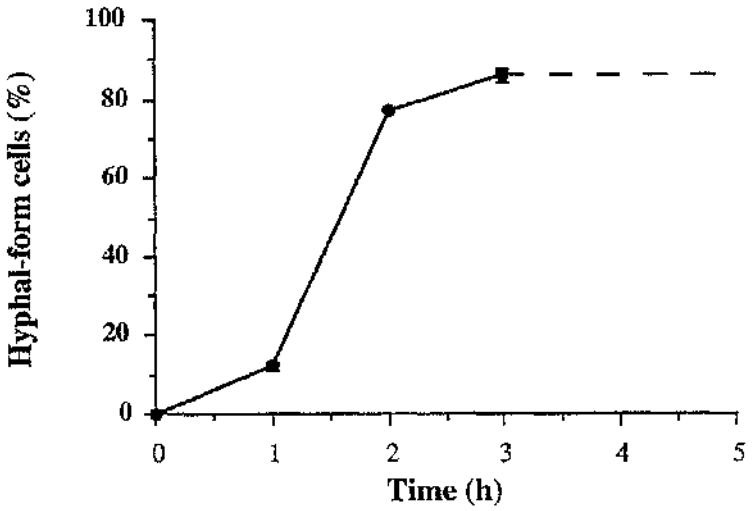
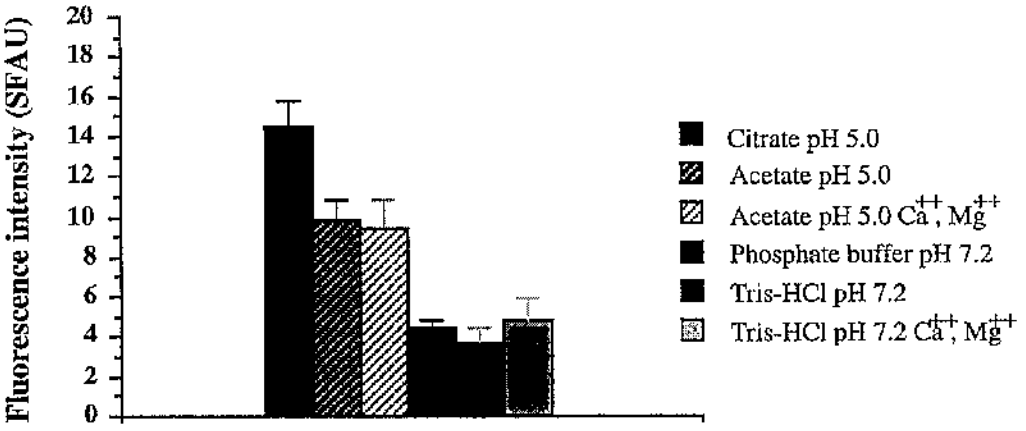


Figure 30. Spectrofluorimetry of *C. albicans* : Effect of different buffers, pHs and Ca^{++} and Mg^{++} on binding of the fucose probe by the hyphal-form cells



confirmed that citrate buffer at pH 5.0 was most suitable for binding of the fucose probe to the hyphal-form cells.

4.3.3. Image Analysis of Hyphal-Form Cells

4.3.3.1. Large scale images (100 x 100 pixels)

The previously presented fluorescence microscopy photographs and image analysis prints showed that the fucose probe bound to hyphal-form cells. Individual hyphal-form *C. albicans* were separated into yeast and germ tube portions by computer-assisted image analysis so as to measure the fluorescence intensity of the bound probe on both portions of the cell. The hyphal-form cells were labelled with the fucose probe (300 $\mu g\ ml^{-1}$) at 37°C for 1 h according to 4.2.2. The cells were washed twice with citrate buffer and resuspended in 0.2 ml of the same buffer. The image analysis was done with the H100 program which creates big squares of 100 x100 pixels and allowed the fluorescence intensity of each portion of the cell to be measured. This procedure was applied to 10 cells to get a representative result.

Note : All of the figures in section 4.3.3 are presented together after page 125.

Figure 31 shows that there was no difference between the two portions in their fluorescence intensity on Day A, while on Day B the germ tube portions were more strongly labelled, but not significantly so.

A problem that emerged during this work was the destruction by blue light, of the fluorescent probe during long exposure. This is shown in Figure 32 where the fluorescence intensity decreased when the exposure was lengthened to around one minute. As a result of this observation, efforts were made to keep all exposures short, preferably less than 20 sec.

4.3.3.2. Small-scale images (10 x 10 pixels)

The differences in the fucose probe binding to the yeast cell and germ tube portions of hyphal-form *C. albicans* were also explored with the small-scale (10 x 10) program.

For this purpose, on each of 3 days, 30 hyphal-form cells under bright field and under blue light were captured and stored on the hard disk of the computer to prevent both quenching and fluorescence decay. The other purpose of this experiment was to compare the tip of the germ tube portion with the other areas on the hyphal-form *C. albicans*. This was done by creating three small areas on each germ tube and on each yeast cell portion and on the background. These measurement were carried out on 90 cells over three days, on each day 30 cells being recorded and stored on the computer. Fluorescence intensity of the fucose probe in each area on the hyphal-form cells was measured according to Figure 10 at page 69. Their backgrounds were substracted. For statistical analysis, the areas on each hyphal-form cells were numbered starting from the tip of the germ tube portion (1), middle (2), end (3) and the yeast cells portion (4, 5, 6) as shown in Figure 10. The 90 readings were summarized on MINITAB and the output presented as boxplot diagrams (Figure 33A) and bar charts (Figure 33B). Both types of diagram indicate a) that the three germ-tube areas (Areas 1, 2, 3) had more fucose probe bound than the yeast-cell areas (Areas 4, 5, 6) and b) that on the germ tube, the highest intensity was not at the tip, but in the adjacent region (Area 2).

Since the differences in the fucose-probe binding between germ-tube and yeast-cell portions were fairly small, further statistical analysis was performed. Each of 30 hyphal-form cells on each of the 3 days were t-tested for the two portions (germ tubes versus yeast cells) and 90 t-values so obtained were presented in Figure 34. This fairly complicated diagram shows a) that results on the 3 days were similar in general pattern; and b) that there were many more positive t-values, than negative ones, indicating a higher fluorescence intensity on the germ tube portions than on the yeast cells. Moreover c) out of the 90 t-tests done, 36 reached the P = 5% level of significant and 19 reached the P = 1% level. On the other hand, only 3 of the negative t-test (i.e. yeast cell portion more fluorescent than germ tube portion) reached P = 1%, while 8 were at P = 5% or greater. The overall conclusion from these detailed analysis was that the germ tube portion of hyphal-form *C. albicans* was capable of binding a significantly higher amount of the fucose probe than the yeast-cell portion.

4.3.3.3. Comparison of five different probes with small-scale images

Similar experiments were then performed with fluorescent probes containing sugars other than fucose, which were run in parallel as known positive controls. These experiments were done on two days. For each probe, 10 hyphal-form cells were captured and stored each day. Fluorescence intensities, with backgrounds subtracted, were recorded as before and Students' t-test applied to interprete the results (Figure 35A and B). Inspection of these figures show that only the fucose and mannose probes bound preferentially to germ tube portions of hyphal-form *C. albicans.* Binding of the other probes (glucose, galactose, lactose) on both portions was so weak that good data were difficult to obtain. For example, the focus of the image was not as good as with the fucose probe. The t-test showed no preferential binding of the glucose, galactose and lactose probes.

According to this data, bar charts were produced to compare fluorescence intensity of the probes on the germ tube portions of the hyphal form cells on two days. This result also indicated that fucose probe bound more than the other probes to germ tube portion of the cells in Figure 36A and B.

4.3.4. Applications of Flow Cytometry

For further studies of the binding of the fucose and other probes to hyphal-form *C. albicans*, use was made of a flow cytometer, available through the courtesy of the Department of Immunology, University of Glasgow. This instrument has the following advantages over spectrofluorimetry or image analysis:

a) rapid reading and population analysis of large number of individual cells;

b) suitability for studies of inhibition of binding of the fucose probe by sugars;

c) no requirement for pronase treatment.

The hyphal-form cells were labelled with the fucose and other probes as before (sections 4.3.2) except that the final suspension was diluted 1 : 10 and dispersed as

Figure 31. Image analysis of *C. albicans* by fluorescence microscopy: Measurement of fluorescence intensity on yeast cell and germ tube portions of the hyphal-form cells labelled with the fucose probe on two days (**A** and **B**). H 100 image analysis program was used. Each bar shows the mean fluorescence intensity of portions of 10 cells and error bars represent SEM

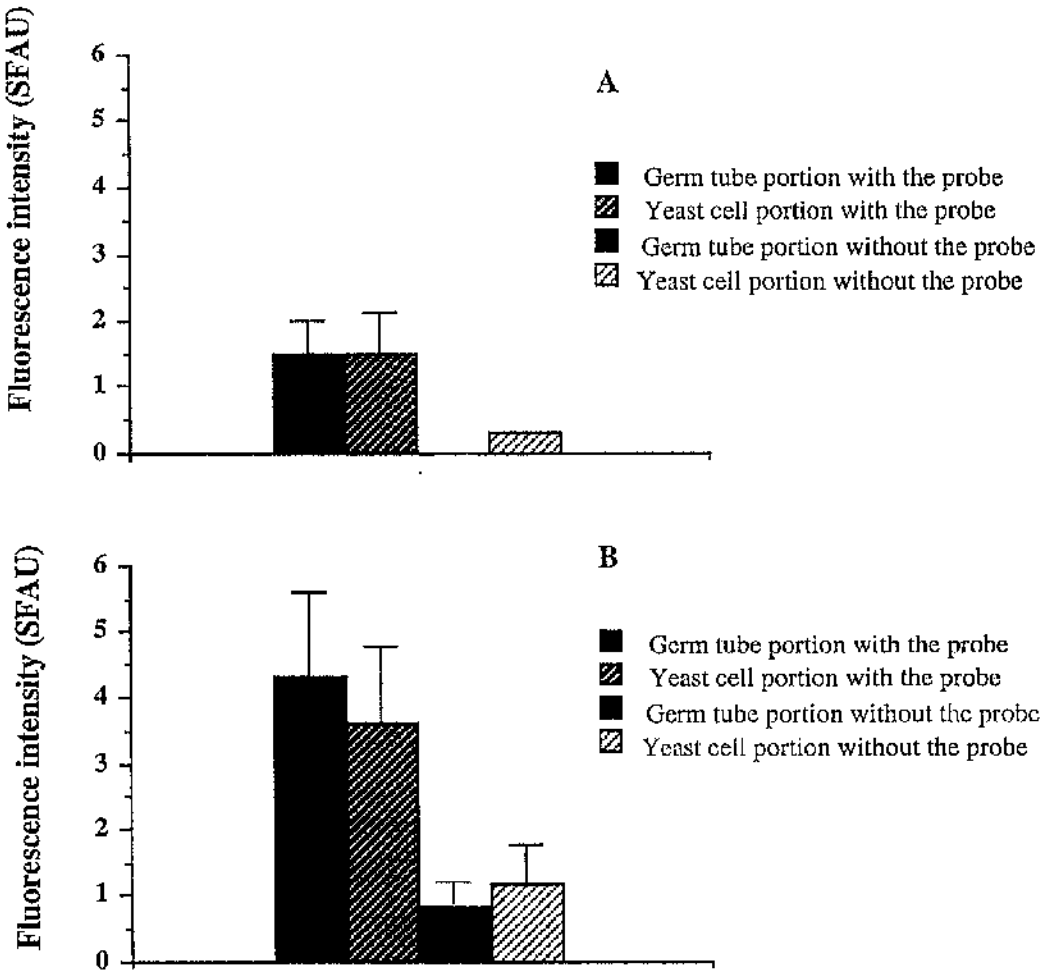


Figure 32. Image analysis of *C. albicans* by fluorescence microscopy: Measurement of fluorescence intensity of yeast cells and germ tube portions of two hyphal form cells on two different days (**A** and **B**) labelled with the fucose probe, and their backgrounds during exposure time of blue light. H100 image analysis program was used

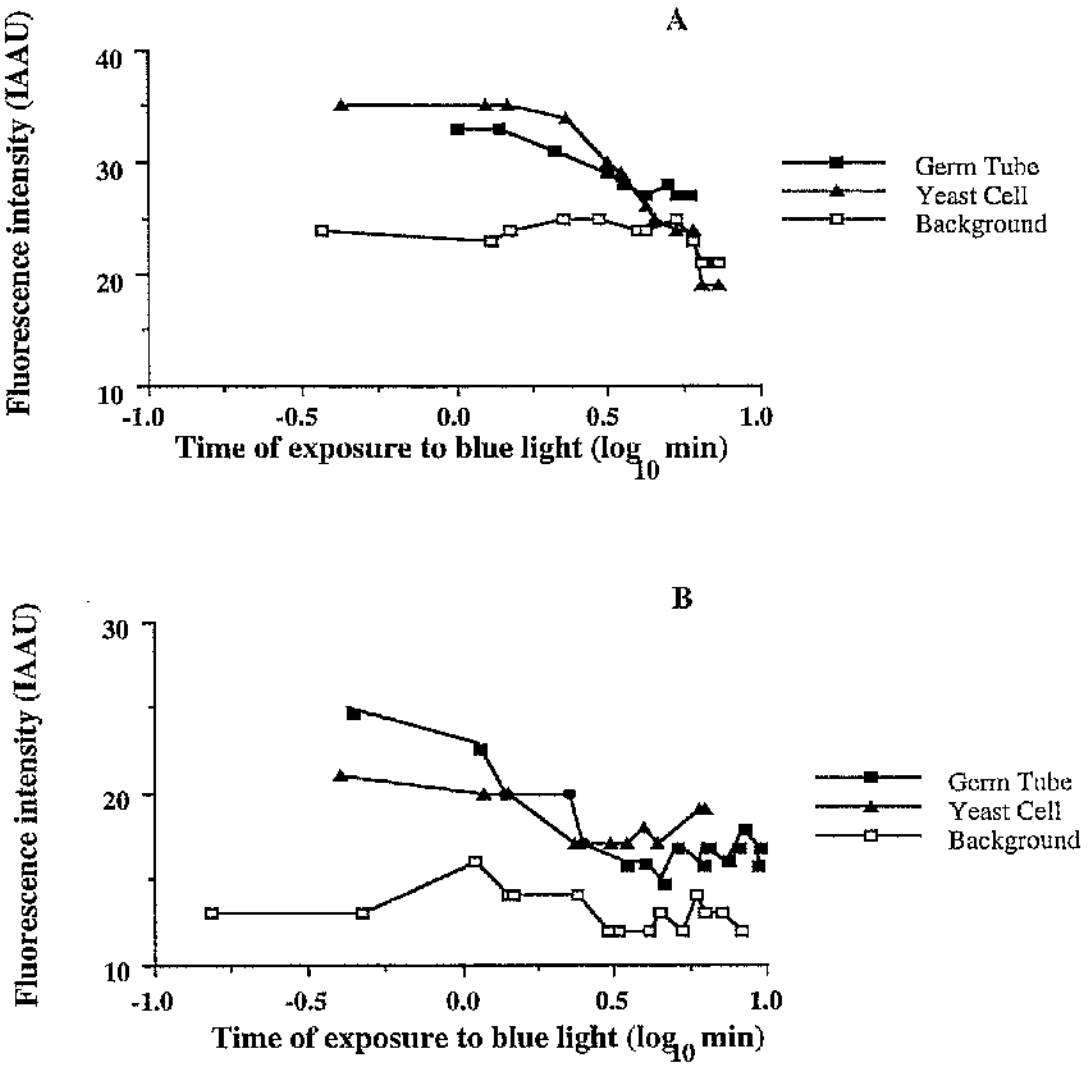
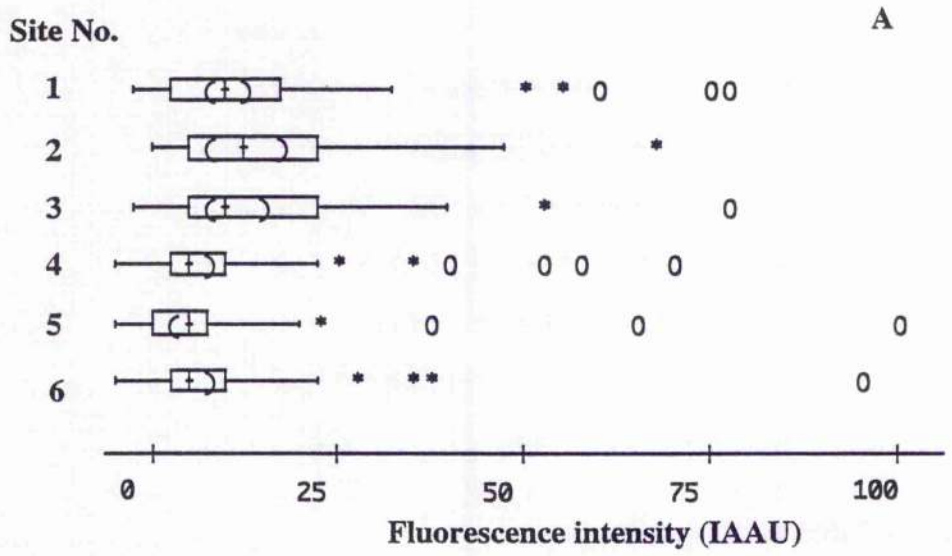


Figure 33. Image analysis of *C. albicans* : **A** Boxplot summaries of individual site measurements from 90 hyphal-form cells; each plot shows the truncated range, median, quartiles, 95% confidence intervals and outlier values; **B** Bar chart summary of truncated means and SEM for each site; truncation involved removing the bottom and top 5% of observations.



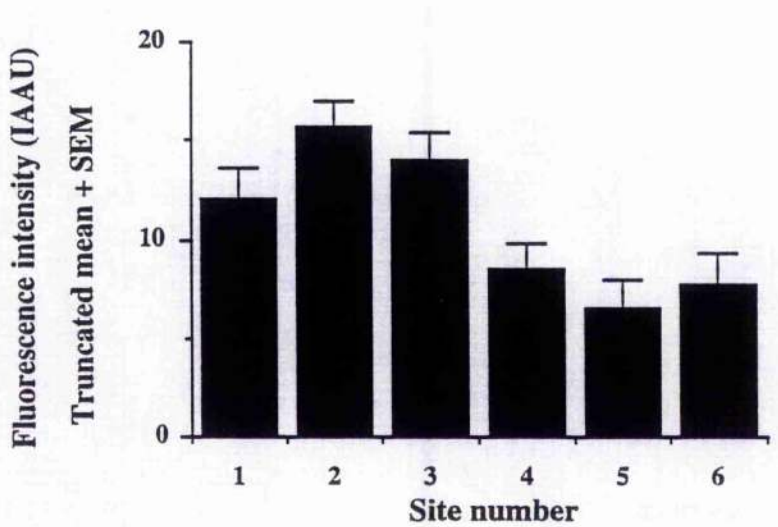


Figure 34. Image analysis of *C. albicans* by fluorescence microscopy : Summary of statistical analysis of t-values for comparison of means of fluorescence intensity of yeast cell (**P**) and germ tube portions (**Q**) of the hyphal form cells labelled with the fucose probe. The vertical dotted lines are at $\pm$ 2.447 and $\pm$3.707, the critical values for P (%)= 5 and 1 respectively

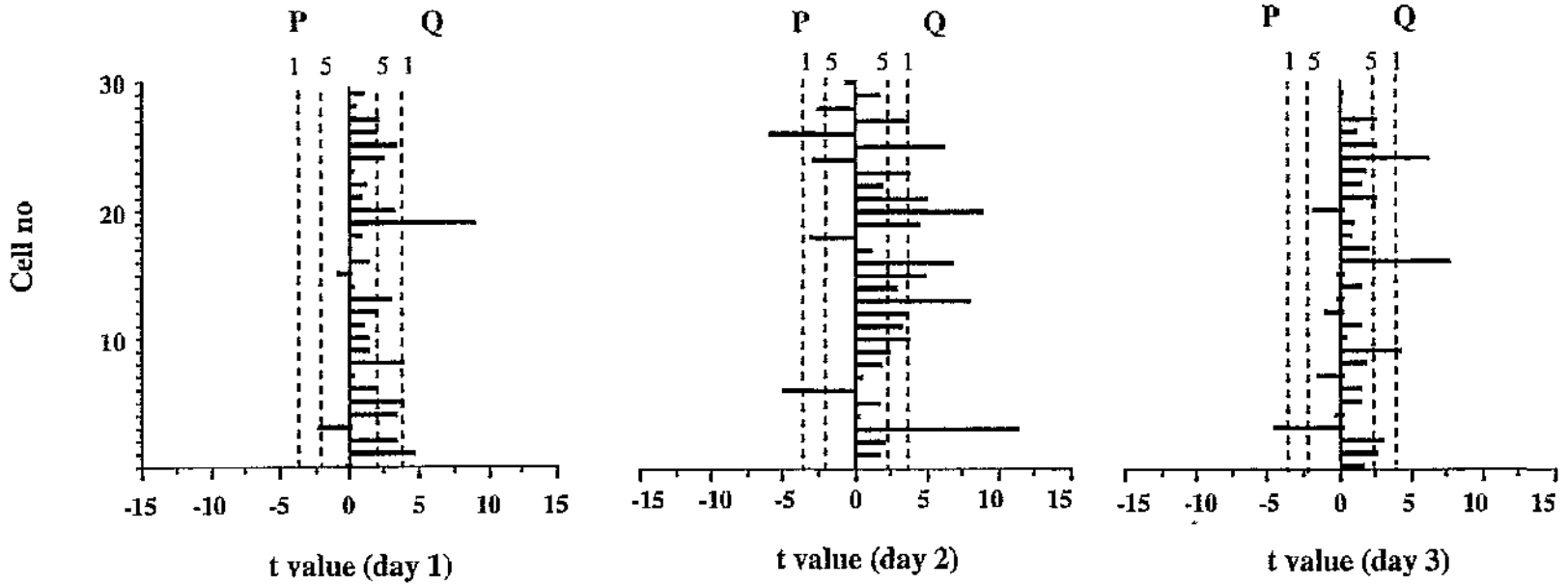


Figure 35A. Image analysis of *C. albicans* by fluorescence microscopy : Summary of statistical analysis of t-values for comparison of means of fluorescence intensity of yeast cell (P) and germ tube portions (Q) of the hyphal-form cells labelled with various probes. The vertical dotted lines are at ± 2.447 and ±3.707, the critical values of t for P (%)= 5 and 1 respectively

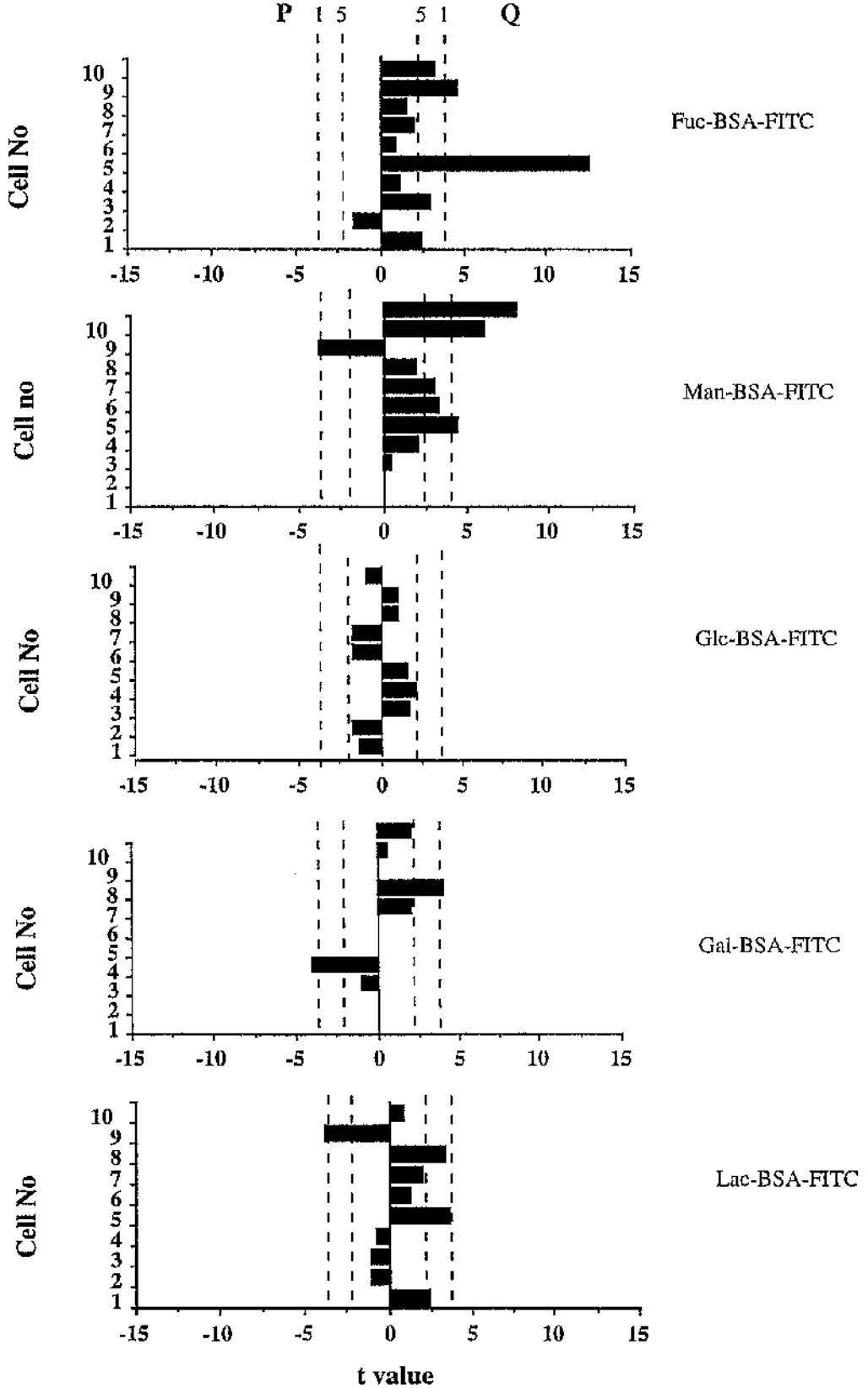


Figure 35B. Image analysis of *C. albicans* by fluorescence microscopy: Summary of statistical analysis of t-values for comparison of means fluorescence intensity of yeast cell (**P**) and germ tube portions (**Q**) of the hyphal-form cells labelled with various probes. The vertical dotted lines are at ± 2.447 and 3.707, the critical values of t for P (%) = 5 and 1 respectively

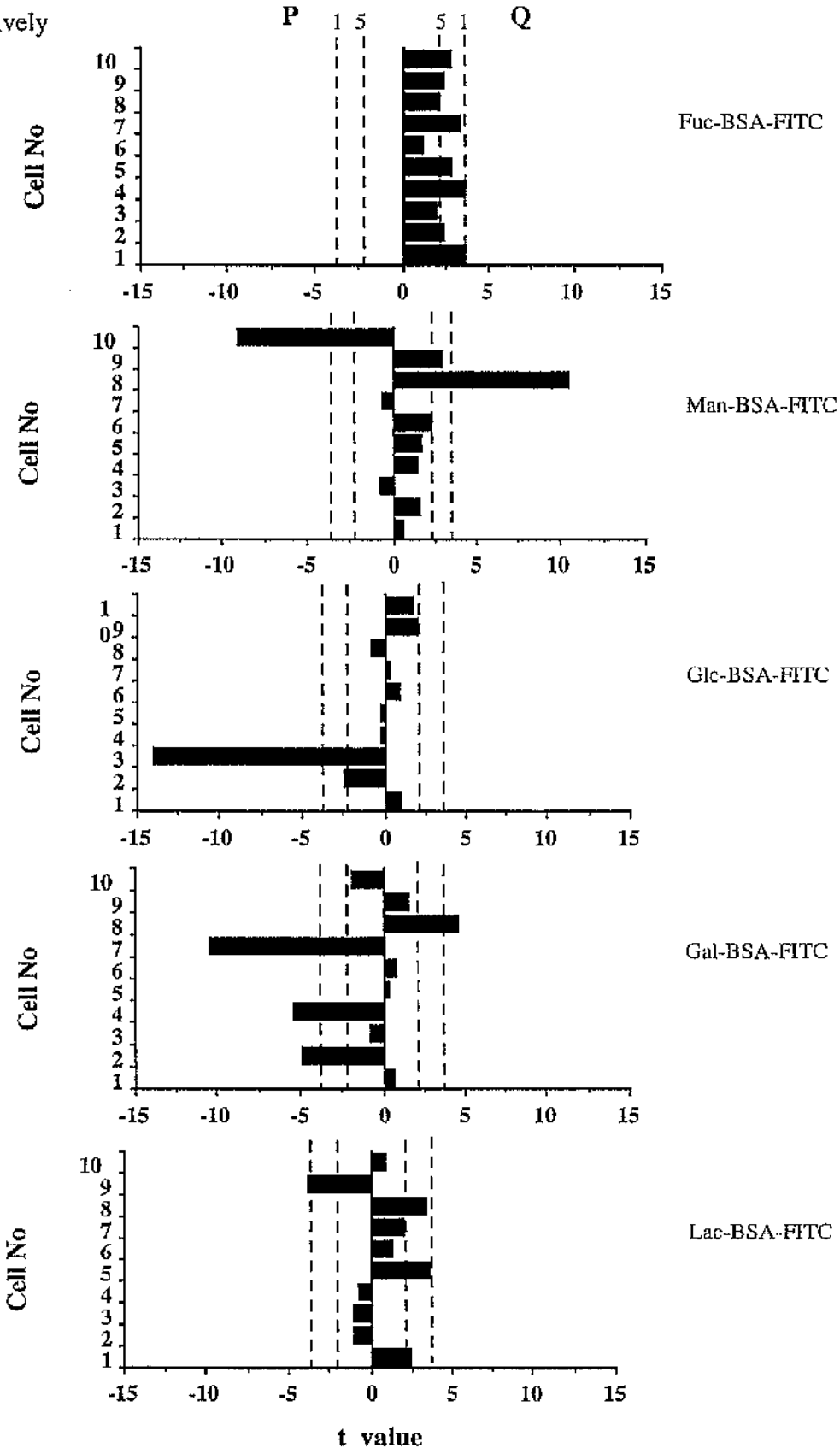
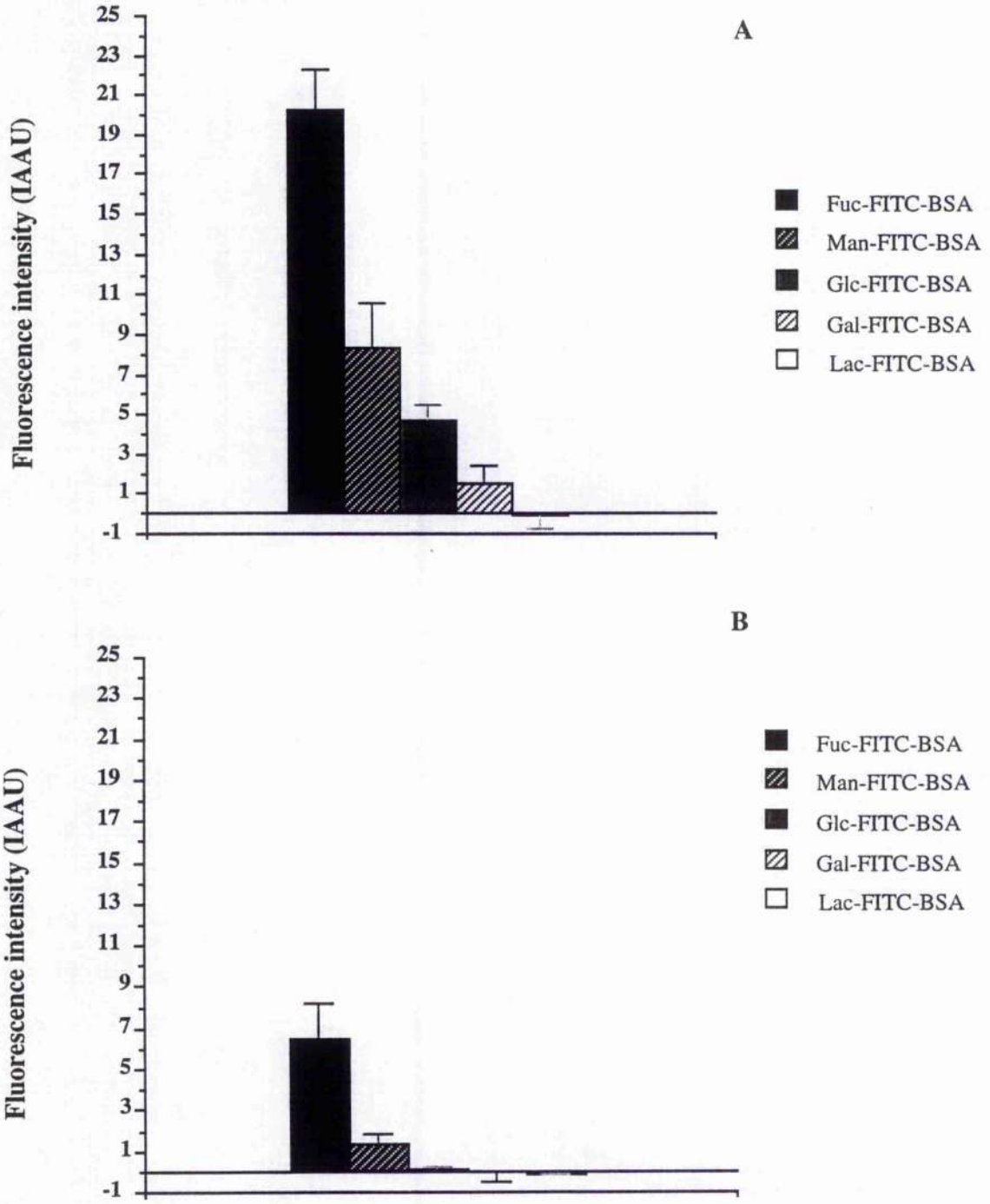


Figure 36. Image analysis of *C. albicans* by fluorescence microscopy : Measurement of fluorescence intensity of the germ tube portions of the hyphal-form cells labelled with five probes on two different days (**A** and **B**)



thoroughly as possible by repeated mixing with the Gilson pipette. This was necessary to minimize yeast and hyphal-forms clumping. For the same reason the reading were taken as rapidly as possible after mixing. In the typical run 3000 cells were passed through the instrument.

Note: All of the tables and figures in section 4.3.4 are presented together after page140.

4.3.4.1 Light scatter patterns during germ tube production

The record produced by the flow cytometer was in two forms: dual-parameter contour plots as in Figure 37, and single-parameter histograms, as in Figure 38. The former provides a measure of the distribution of cell sizes and densities; for example Figure 37 shows elongation of the shape during incubation for 2 h as germ tubes are produced by the yeast cells. The elongation is represented by the diagonal lengthening of the contours with time, and the progressive strengthening of the detected image at the top right corner of each plot. These images may be aggregated cells. Figure 37 was produced with unlabelled *C. albicans*.

4.3.4.2. Binding of the fucose and lactose probes during germ tube production

The single-parameter histograms, such as Figure 38, were better suited for detection of specific fluorescence with a probe, because they allowed subtraction of the background fluorescence from unlabelled cells that were run in parallel. Thus Figure 38 shows that during germ tube formation over 2 h in 199 Medium, the peak fluorescence of fucose-probe-labelled cells moved progressively to the right, whereas the fluorescence of lactose-probe-labelled cells remained associated with background. The abscissa on Figure 38 is in FCAU, which stands for flow cytometry arbitrary units, and is a measure of fluorescence intensity.

Experiments such as that in Figure 38 were repeated several times and the original records stored in Appendix 6. For summary purposes, the collected results of

such experiments were summarized in bar charts as Figure 39. This figure shows that during germ tube production of *C. albicans*, there was a progressive increase in binding of the fucose probe, whereas the lactose-probe-treated cells remained similar to background (unlabelled) cells. These results indicated that the fucose probe a) bound to yeast cells of *C. albicans* and b) the amount of bound label increased during germ tube formation.

In order to produce the bar charts from Figure 38, it was first necessary to choose boundary markers on the FCAU scale as shown in Figure 12 so as to best separate unlabelled cells which had lower fluorescence intensity. In this figure, boundaries were chosen at 1.76 and 4.0 on the $\log_{10}$ FCAU scale and then used in the Lysis II statistics package to generate the bar charts and SEM values.

In figure 39A the fluorescence is expressed in terms of the percentage of the labelled cells that falls within the markers 1.76 and 4.0 FCAU, while Figure 39B shows the intensity in FCAU that falls within the marker limits. For most purposes the top chart was most useful but in a few cases additional information was obtained from the fluorescence intensity diagrams.

An approach similar to that described in this section was used to investigate the effect of a wide selection of variables that might influence probe-binding during germ-tube formation. An index to those flow-cytometry experiments is provided in Table 8 which summarizes the location of the results and their subsequent presentation and analyses.

4.3.4.3. Effect of growth in 199 Medium at different pHs and temperatures

Further comparisons of the fucose-probe labelling of *C. albicans* yeast and hyphal-form cells were made by alterations of pH and temperature. Thus in 199 Medium at pH 6.7 and Glucose-Glycine Medium pH 7.2, the dual-parameter contour plots in Figure 40 (top) show the expected formation of germ tubes by the extended fluorescence towards the top right corner of diagrams. Reducing the temperature to 22°C, greatly

diminished germ tube production (middle diagrams), while at pH 4.0 and 37°C the cells remained in the yeast form (bottom diagrams). This results were also confirmed by counting of yeast cells according to production germ tube under bright field microscopy (Table 9).

The corresponding single-parameter histograms (Figure 41) provide an alternative presentation with the background values from unlabelled cells superimposed. Statistical analyses were done on the cell populations between boundary markers 1.76 and 4.0 on the FCAU scale and are presented as bar charts in Figure 42. These figures show that the percentage of cells undergoing germ tube production conditions at pH 6.7 and 37°C increased up to 2 h, which confirmed the previous experiment. The percentage of the yeast cells under the same incubation conditions over 2 h was lower than the cells undergoing germ tube production. Lower percentages and fluorescence intensities of the cells were obtained at pH 4.0 and 37°C up to 2 h. Fluorescence intensities of the cells under germ tube and yeast cell production conditions at 37°C and 22°C respectively were increased at 1 h and then decreased at 2 h.

4.3.4.4. Effect of growth media

A comparison was made of Glucose-Glycine (GG) Medium pH 7.2, with 199 Medium pH 6.7, for allowing *C. albicans* to express fucose-specific adhesins during germ tube formation. In order to check percentage of germ tube production by *C. albicans* in two media at different pHs, the cells were also counted under bright field microscopy (Table 10). The single-parameter histogram in Figure 43 representing germ tube formation show that almost identical results were obtained in the two media. However some differences did emerge in the statistical analysis, and Figure 44 (bottom diagrams) indicates that at 2 h the fluorescence intensity was significantly higher in cells grown in GG medium over those in 199 Medium.

4.3.4.5. Comparison of different probes on the yeast and the hyphal-form cells

The histograms in Figure 45 show that the yeast-form cells were significantly labelled (above background) only by the fucose probe. Thus the other probes - mannose, glucose, galactose and lactose - gave histograms in which labelled and unlabelled cells were coincident. However a different picture emerged with the hyphal-form cells. Here both the fucose and mannose showed well displaced histograms, while the glucose and galactose probes gave histograms with small displacements of the labelled cells over the unlabelled. Only with the lactose probe were the histograms essentially coincident. These findings were strengthened by the bar charts (Figure 46) which confirm the fucose probe as having the highest specificity of binding for both yeast and hyphal-form cells, and the hyphal-form cells binding the other probes in the order mannose > glucose > galactose > lactose.

4.3.4.6. Effect of Ca^{++}, Mg^{++} and EDTA on binding of the fucose probe

The experiments were done in several buffers : citrate and acetate at pH 5.0 and phosphate and tris-HCl at pH 7.2. The Ca^{++} and Mg^{++} were added at 2 mM. To explore the effect of removing traces of Ca^{++} and Mg^{++} that might be present, 5 mM EDTA was prepared in the tris-HCl buffer.

Visual inspection of the result in Figure 47 indicates that binding of the fucose probe to hyphal-form *C. albicans* took place in all 4 buffers at both pHs, and in the presence or absence of Ca^{++} and Mg^{++}. This visual impression is confirmed by statistical analysis (Figure 48) where the bar charts and SEMs all overlap in the high level of binding exhibited. There were minor differences relating to buffer types, pH or divalent cations, but the overall pattern was one of uniformity.

The non-involvement of Ca^{++} and Mg^{++} in fucose-probe binding to hyphal-form cells was further estabished by the EDTA experiment. Figures 49 and 50 show essentially no influence of this chelator on the system.

4.3.4.7. Hyphal-form *C. albicans* labelled with the fucose probe at different temperatures

The labelling procedure with the hyphal-form cells was done at temperatures of 37, 22 and 4°C. Single parameter histograms showed that the labelling was high at 37°C (Figure 51). According to statistical analysis within the marker limits, in Figure 52 the percentage of the labelled cells that the fucose probe bound was higher at 37°C than at 22°C. There was less binding at 4°C. The fluorescence intensity of the cells labelled at 22°C was lower than at 37°C and 4°C

4.3.4.8. Comparison of Fuc-BSA-FITC, Lac-BSA-FITC and BSA-FITC

In this experiment BSA-FITC was used to explore whether the protein part of the probes was involve in binding to the hyphal-form cells. The fucose and lactose-labelled cells were chosen as positive and negative controls respectively. Visual impression of the results in Figure 53 indicated that the BSA-FITC probe bound to the hyphal-form cells as well as did the Fuc- BSA-FITC. Statistical analysis of the cells in the marker limits was done and represented in Figure 54. Percentage of the cells labelled with BSA-FITC was higher than that with the fucose-labelled cells. Fluorescence intensities of these labelled cells were similar. Percentage of the lactose-labelled cells was close to unlabelled cells.

4.3.4.9. Inhibition assay of binding of the fucose probe to the cells

To explore the specificity of the fucose probe for binding to hyphal-form cells, Fuc-BSA was used as a homologous probe for the fucose probe (with FITC). Control experiments were done with Gal-BSA, and with the free sugars (L-fucose and D-galactose). In this experiment, the positive control was the cells labelled with the fucose probe, and the negative control was unlabelled cells. Figure 55 shows that the percentage of labelled cells with fucose probe was lowest after preincubation with Fuc-

BSA than when preincubated with Gal-BSA, L-fucose or D-Galactose. Fluorescence intensity of the fucose probe-bound cells did not show marked changes with or without Fuc-BSA or the simple sugars L-fucose and D-galactose. But the cells with Gal-BSA showed high fluorescence intensity (Figure 56).

4.3.4.10. Binding of the fucose probe by wild type *C. albicans* and its germ-tube deficient mutant

To confirm the increasing binding of the fucose probe during germ tube production, wild-type strain MEN and its germ-tube deficient mutant MM2002 were grown in 199 Medium (pH 6.7) at 37°C and analysed after labelling with the fucose probe. Diagrams as dual parameter contour plots are represented in Figure 57. Elongation of wild type-germ tubes during the incubation priod which is up to 2 h was clear, with the contour plots (top). The patterns are consistent with strain 2346 as shown in Figure 37. The mutant strain did not show any elongation of the cells during the same incubation period. Both strains were also counted under bright field microscopy to check percentage of germ tube production (Table 11). The correlation of germ tube production and binding of the fucose probe was visible in Figure 58. Statistical analyses of both strains were done and represented as bar charts and SEMs (Figure 59). The wild-type strain exhibited high levels of fucose probe binding at 2 h incubation time, but the percentage with the mutant strain did not increase (A). Fluorescence intensity of the wild-type at 2 h was higher than with the mutant strain (B). Fluorescence intensity of the wild-type strain at 1 h was higher than that of the mutant strain at 0 and 2 h.

Table 8. Summary table of flow cytometry results and analyses

Binding experiments of *C. albicans* labelled with the fucose probe in citrate buffer at pH 5.0	Location of results and analyses		
	Flow cytometer out put: Figure No	Analytical data: Appendix No 6.	Summary bar chart: Figure No
Comparison with the lactose probe during germ tube production	37, 38	1	39
Medium 199 at different pH and temperatures during germ tube production	40, 41	2	42
Effect of growth in different media and pHs during germ tube production	43	3	44
Comparison with various probes on the hyphal and yeast form	45	4 A, B	46
Effect of acetate buffer with and without Ca^{++} Mg^{++} at pH 5.0	47	5A	48
Effect of phosphate and tris-HCl with and without Ca^{++} Mg^{++} at pH 7.2	47	5B	48
Effect of tris-HCl with and without EDTA at pH 7.2	49	6	50
Effect of different temperatures on the binding	51	7	52
Comparison with the lactose probe and FITC-BSA	53	8	54
Effect of Fuc-BSA, Gal-BSA , L-Fucose and D-Galactose	55	9	56
Wild type (strain MEN) and its germ-tube-deficient mutant (strain MM2002) during germ tube production	57, 58	10	59

Table 9. Germ tube production by *C. albicans* in 199 Medium at different pHs and temperatures. SEM represents five fields counted on two different days

	Percentage germ tubes (± SEM) at		
Time (h)	37°C	22°C	4°C
0	0.0	0.0	0.0
1	25.5 ± 9.8	0.0	0.0
2	78.6 ± 8.2	0.0	0.0

Table 10. Germ tube production by *C. albicans* in different media and at different pH values. SEM represents five fields counted on two different days

	Percentage germ tubes (± SEM) in	
Time (h)	199 Medium	GG Medium
0	0.0	0.0
1	25.5 ± 9.8	28.8 ± 4.6
2	78.6 ± 8.2	79.8 ± 4.1

Table 11. Germ tube production by *C. albicans* wild type (strain MEN) and its germ tube-deficient mutant (strain MM2002) in 199 Medium at pH 6.7 and 37°C. SEM represents five fields counted on two different days

	Percentage germ tubes (± SEM) from	
Time (h)	Wild type	Mutant
0	0.0	0.0
1	35.1 ± 2.4	0.0
2	79.6 ± 1.8	17.3 ± 1.9

Figure 37. Flow cytometry of unlabelled *C. albicans*: Effect of growth in 199 medium at pH 6.7 and 37 °C, showing distribution of cell size and density during germ-tube production. The diagrams are dual parameter contour plots

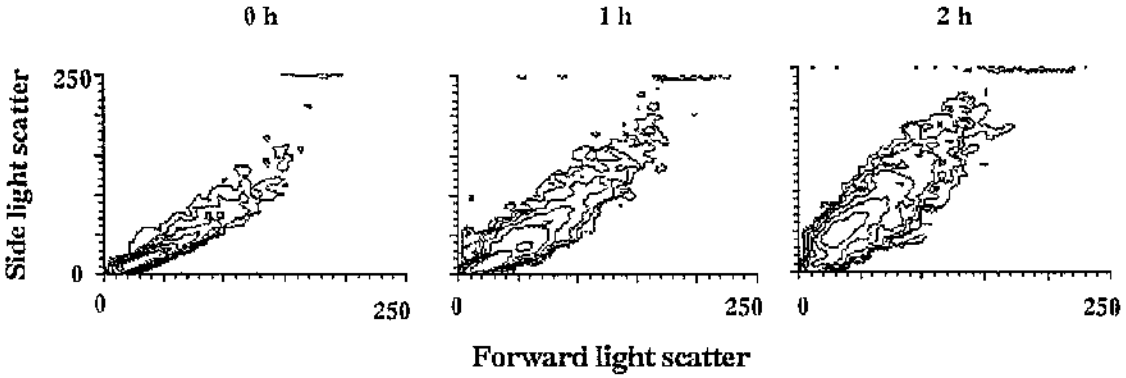


Figure 38. Flow cytometry of *C. albicans*: Comparison of binding of the fucose and lactose probes by the cells during germ tube production. The diagrams show single parameter histograms. The grey line represents unlabelled cells and the black area, the cells labelled with one of the probes

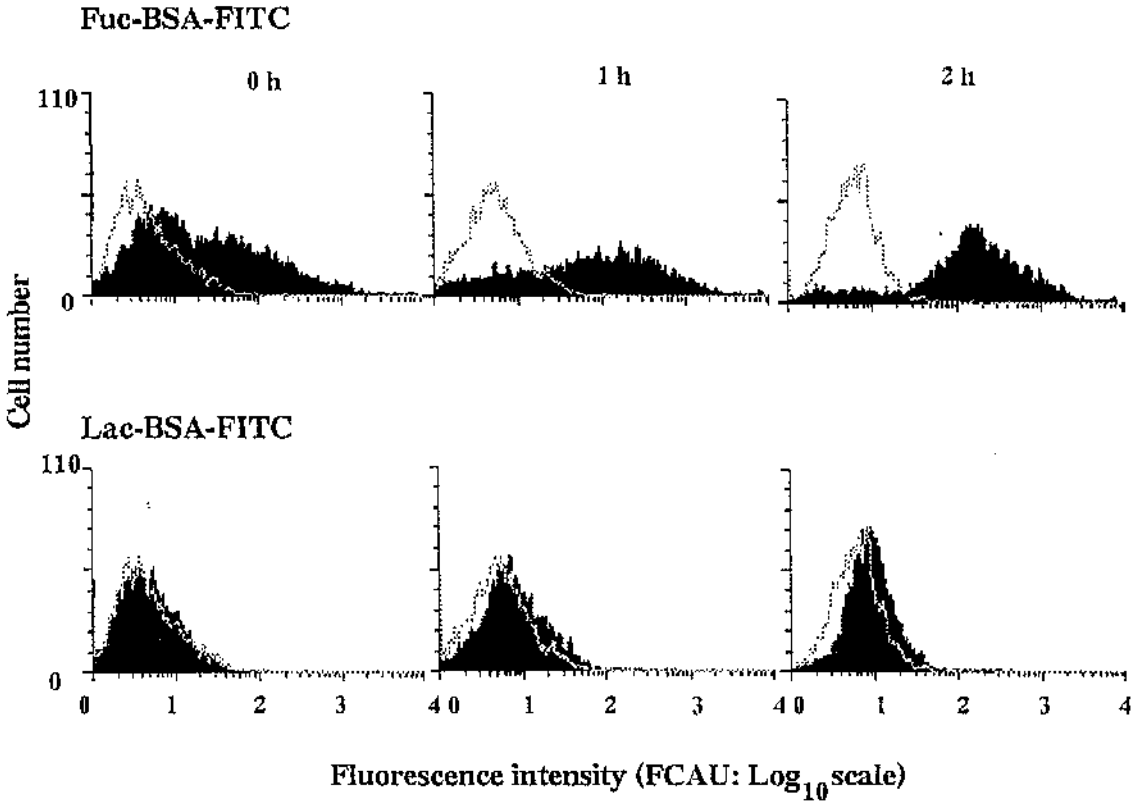
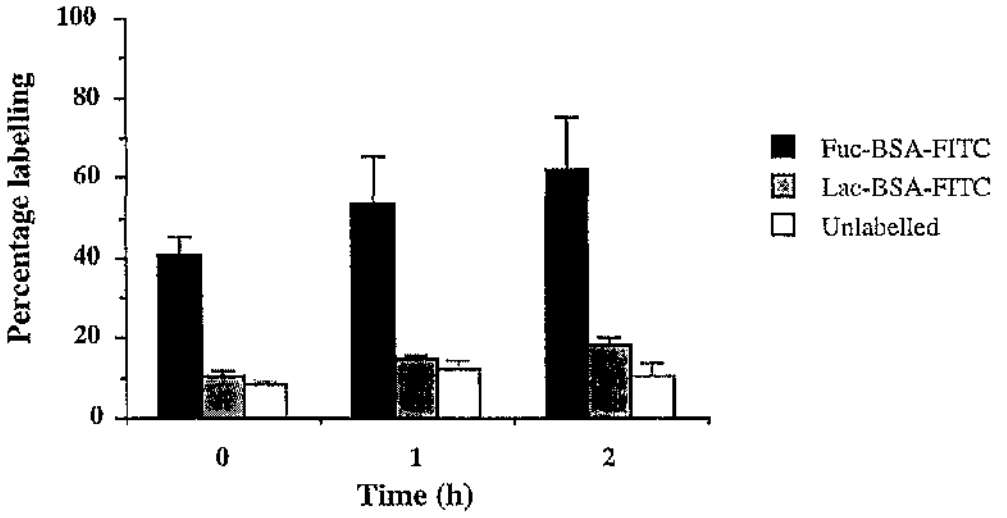


Figure 39. Flow cytometry of *C. albicans* : Summary bar charts to show the binding of fucose and lactose probes by cells during germ tube production. Vertical lines represent SEM

A. Percentage of the cells labelled with the probes, or unlabelled cells, within the marker limits



B. Fluorescence intensity of the cells labelled with the probes, or unlabelled cells, within the marker limits

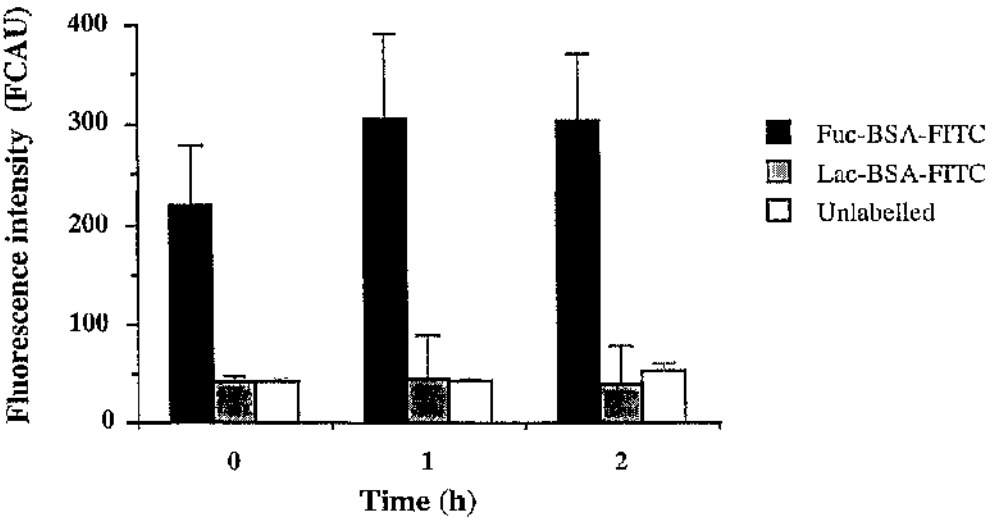


Figure 40. Flow cytometry of *C. albicans* labelled with the fucose probe: Effect of growth in 199 Medium at different pHs and temperatures showing distribution of the cell size and density during germ tube production. The diagrams show dual parameter contour plots

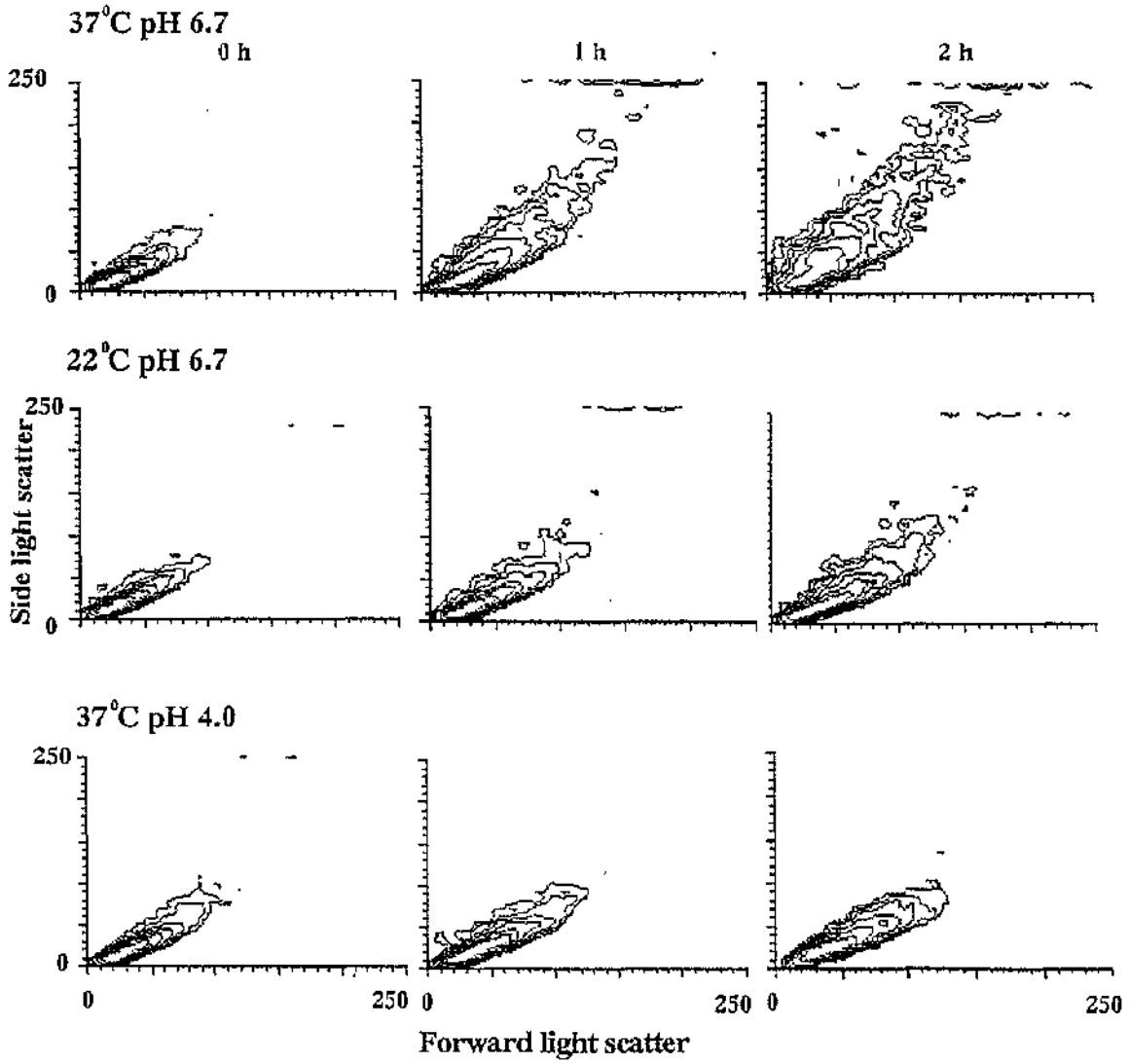


Figure 41. Flow cytometry of *C. albicans*: Binding of the fucose probe by the cells grown in 199 Medium at different pHs and temperatures during germ tube production. The diagrams show single parameter histograms. The grey line represents unlabelled cells and the black area the cells labelled with the probe

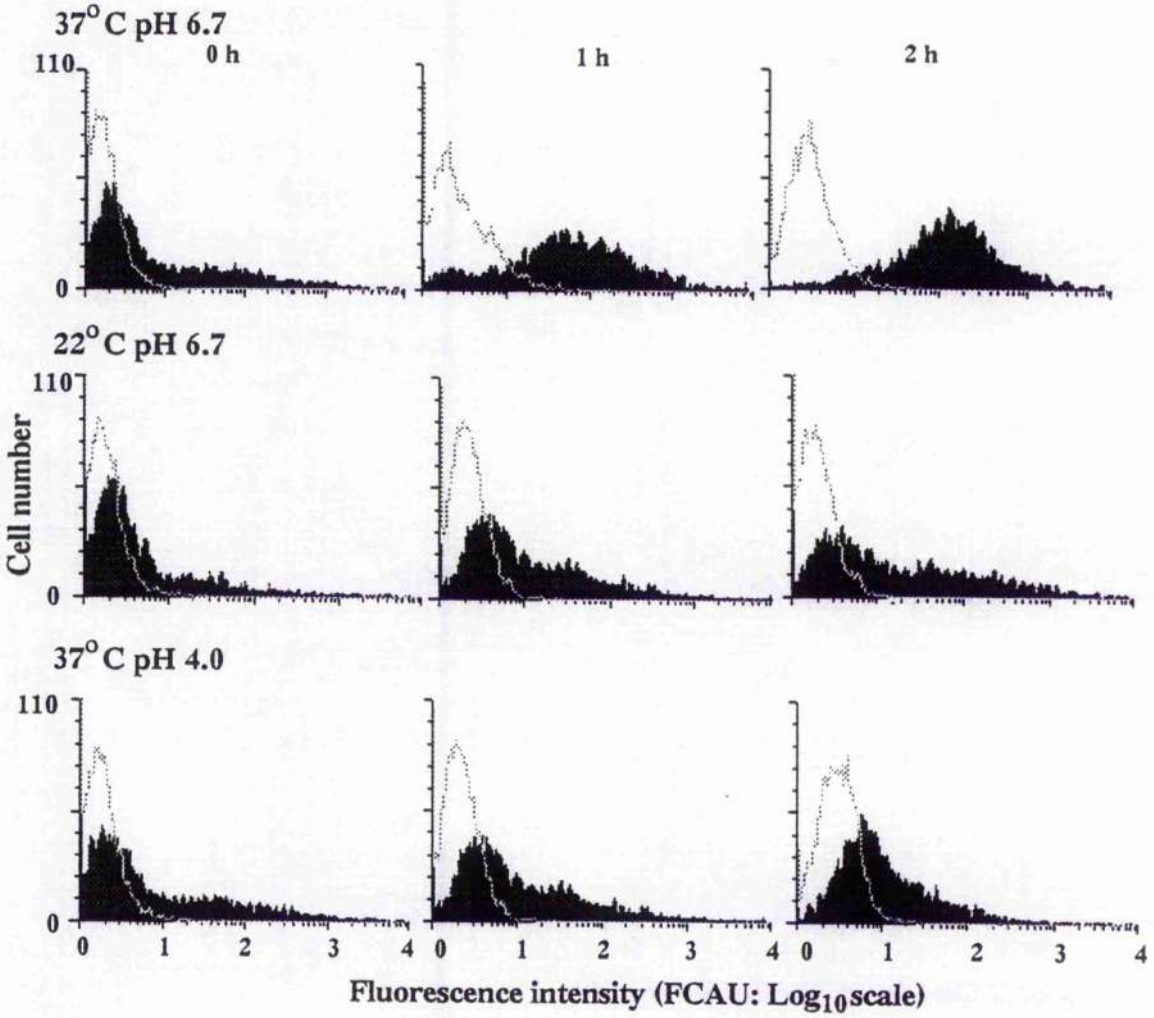
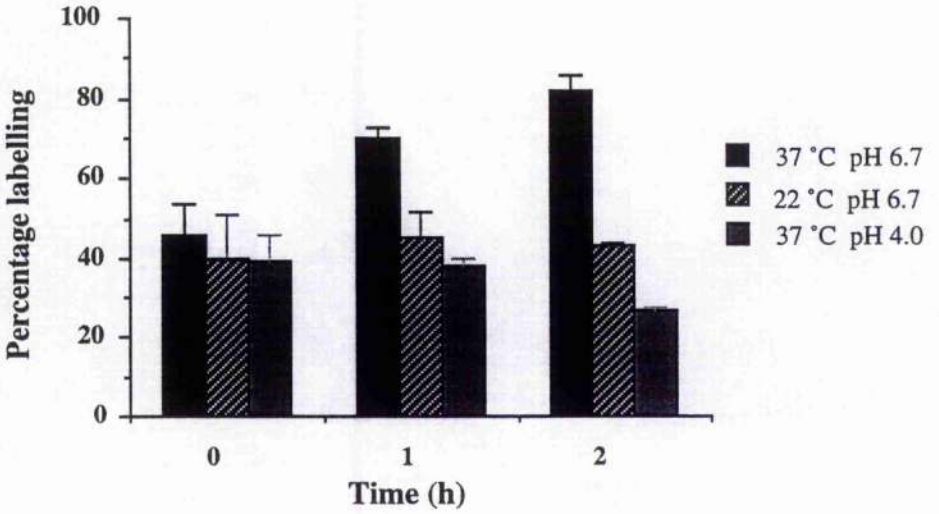
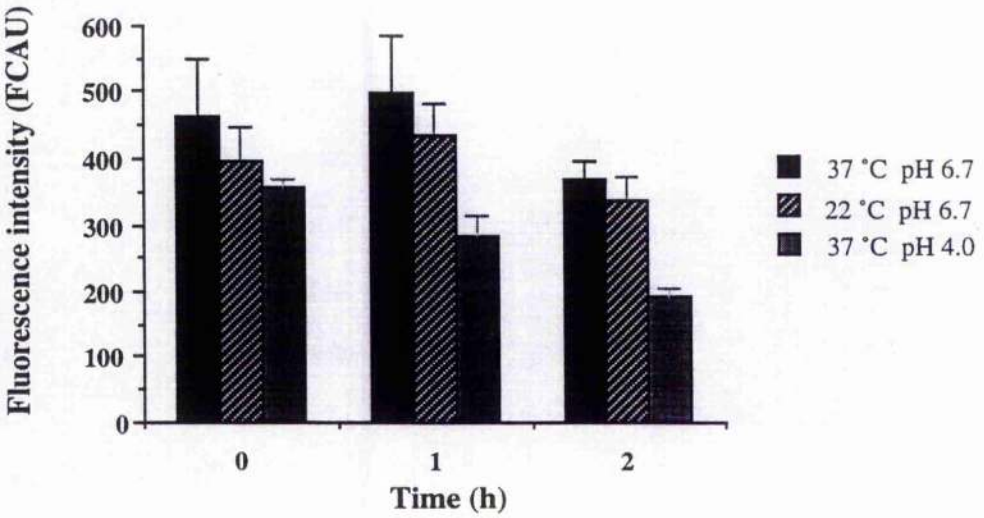


Figure 42. Flow cytometry of *C. albicans* : Summary bar charts for binding of the fucose probe by the cells grown in 199 Medium at different pHs, temperatures during germ tube production. Vertical lines represent SEM.

A. Percentage of the cells labelled with the probe within the marker limits



B. Fluorescence intensity of the cells labelled with the probe within the marker limits



For clarity of presentation, fluorescence intensity of the background readings in the set marker which were very low, are omitted.

Figure 43. Flow cytometry of *C. albicans*: Binding of the fucose probe by the cells grown in different media during germ tube production. The diagrams are single parameter histograms. The grey line represents unlabelled cells and the black area the cells labelled with the probe

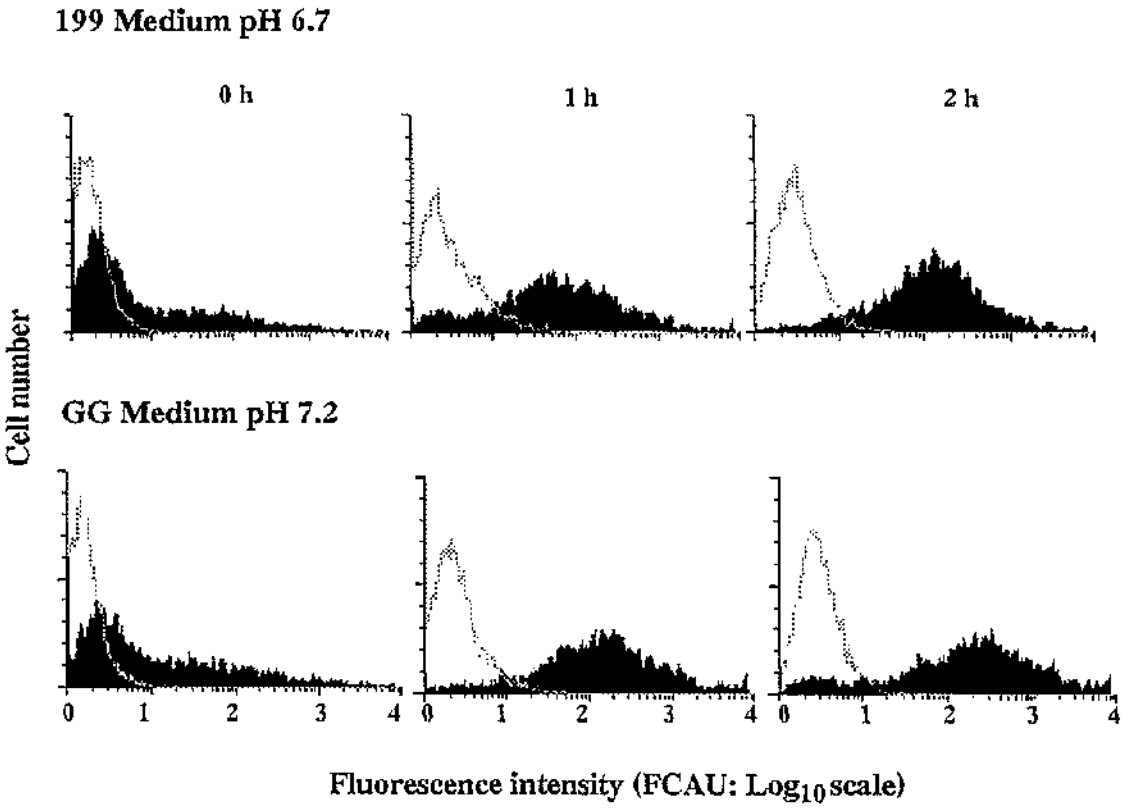
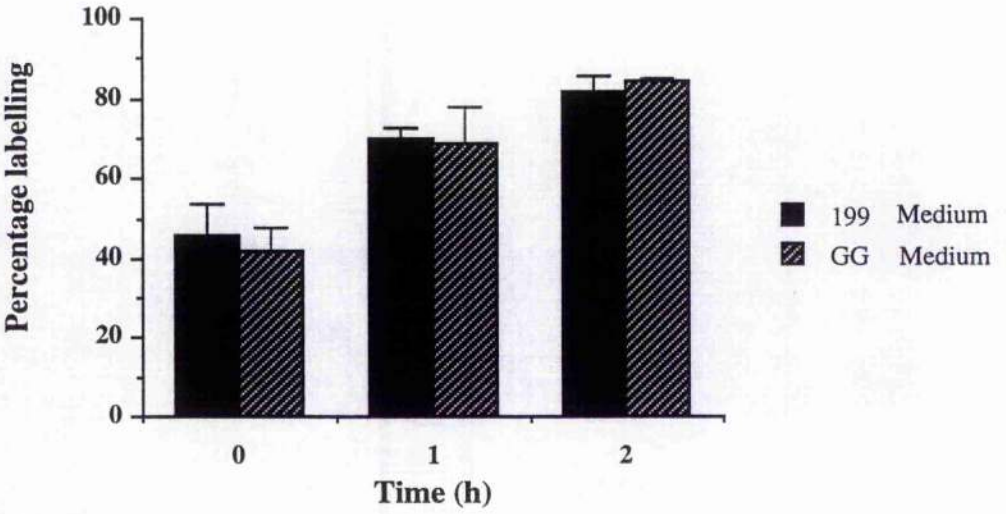
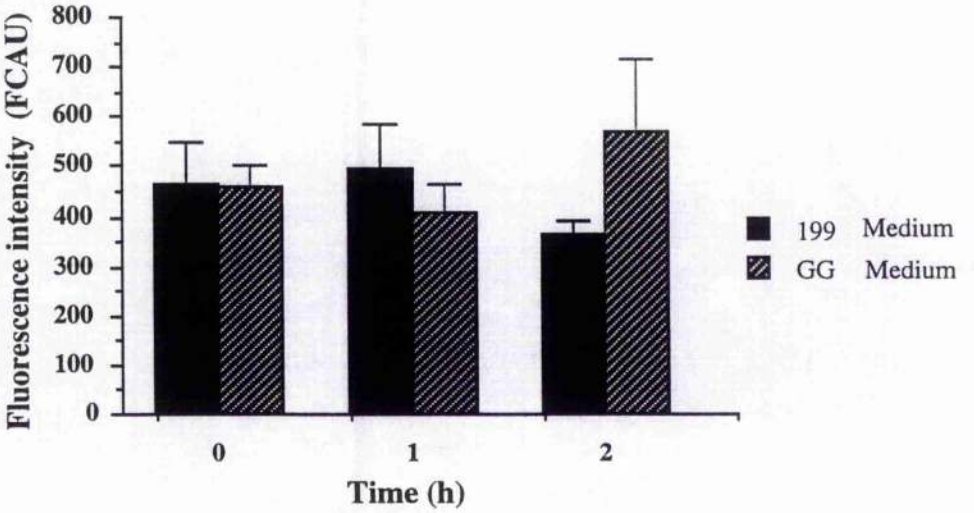


Figure 44. Flow cytometry of *C. albicans* : Summary bar charts to show binding of the fucose probe by the cells grown in different media and pH during germ tube production. Vertical lines represent SEM

A. Percentage of the cells labelled with the probe, or unlabelled, within marker limits



B. Fluorescence intensity of the cells labelled with the probe, or unlabelled, within marker limits



For clarity of presentation, fluorescence intensity of the background readings in the set marker which were very low, are omitted.

Figure 45. Flow cytometry of *C. albicans*: Binding of various probes by the yeast- and hyphal-form cells. The diagrams show single parameter histograms. The grey line represents unlabelled cells and the black area the cells labelled with one of the probes

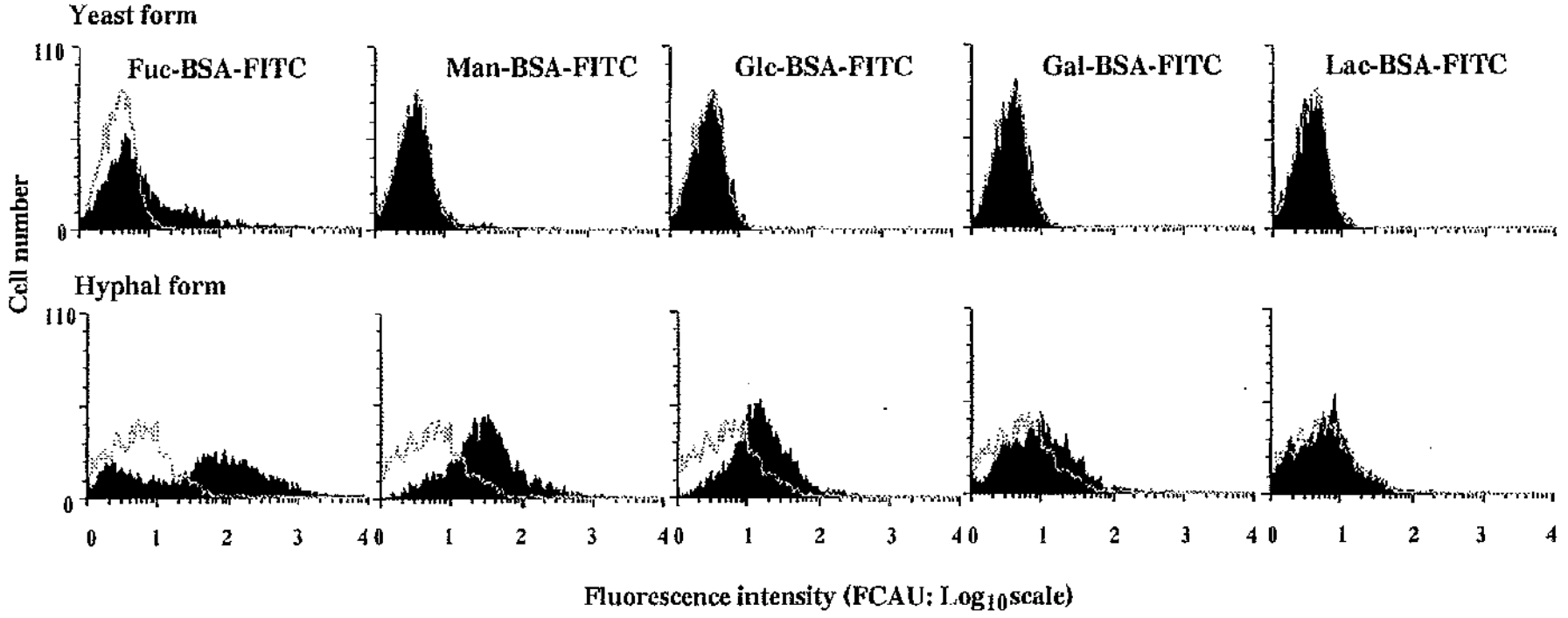
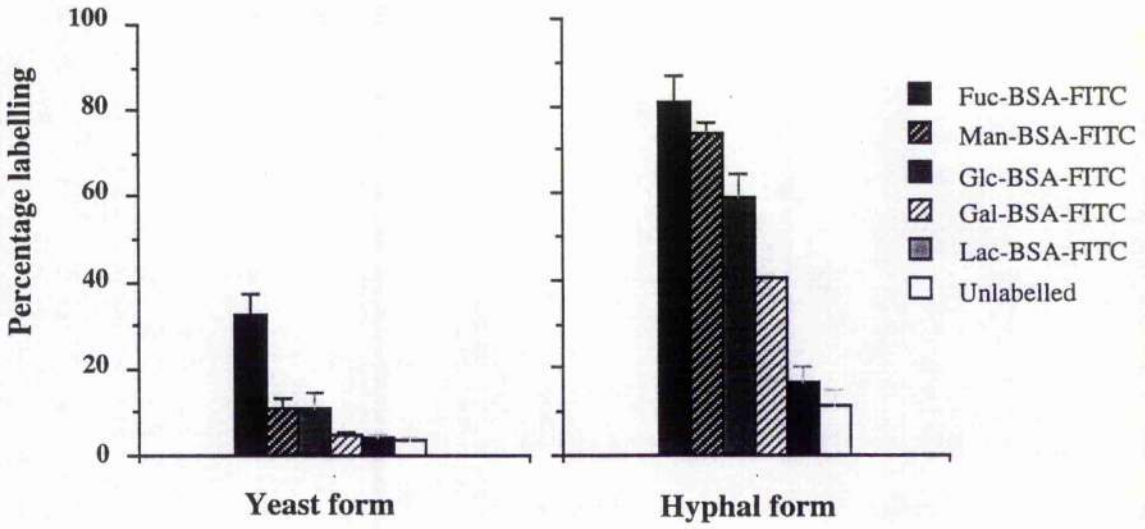


Figure 46. Flow cytometry of *C. albicans* : Summary bar charts to show binding of various probes by the yeast and hyphal forms. Vertical lines represent SEM

A. Percentage of the cells labelled with the probes, or unlabelled, within marker limits



B. Fluorescence intensity of the cells labelled with the probes, or unlabelled, within marker limits

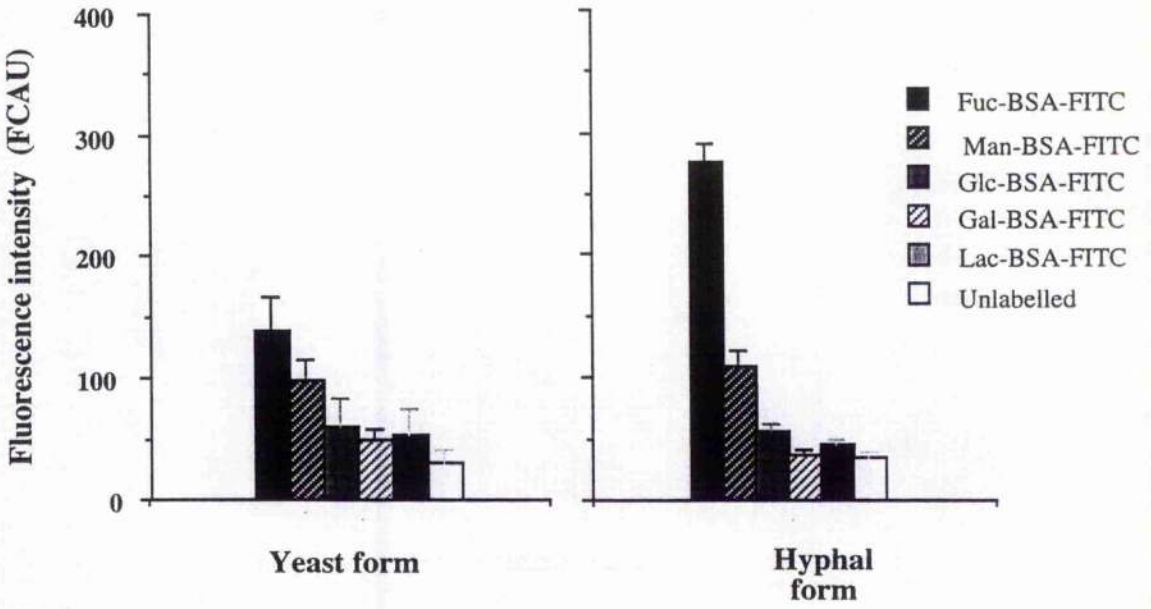


Figure 47. Flow cytometry of *C. albicans*: Binding of the fucose probe by the hyphal-form cells in different buffers at pH 5.0 and 7.2 with and without Ca^{++} and Mg^{++}. The diagrams show single parameter histograms. The grey line represents unlabelled cells and the black area the cells labelled with the probe

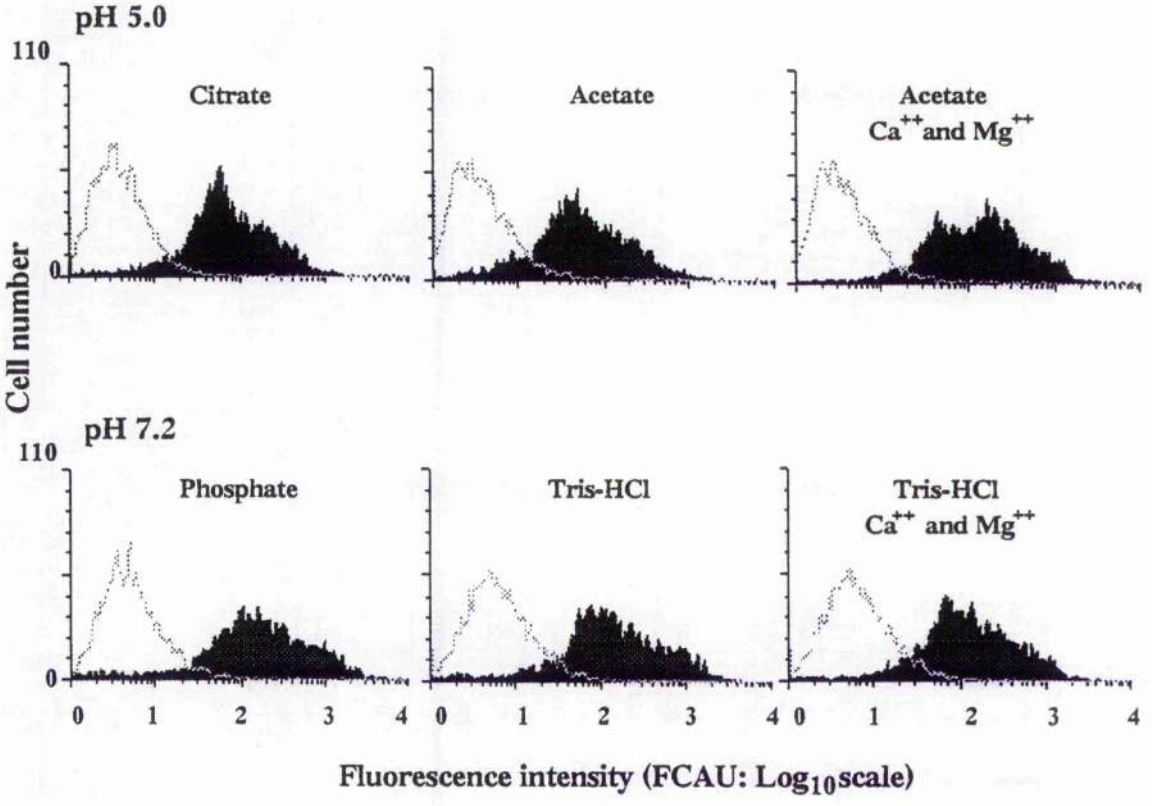
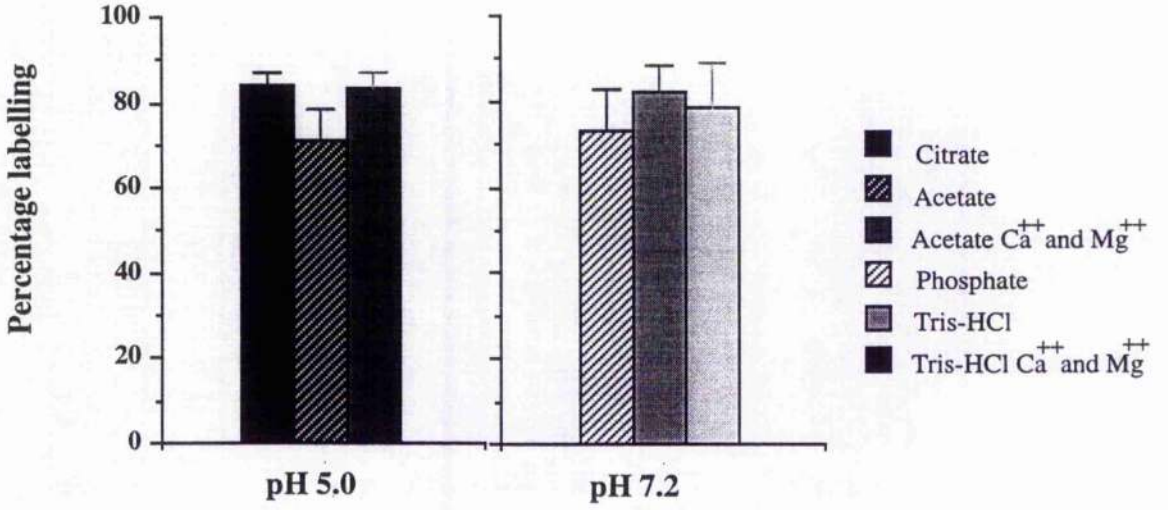
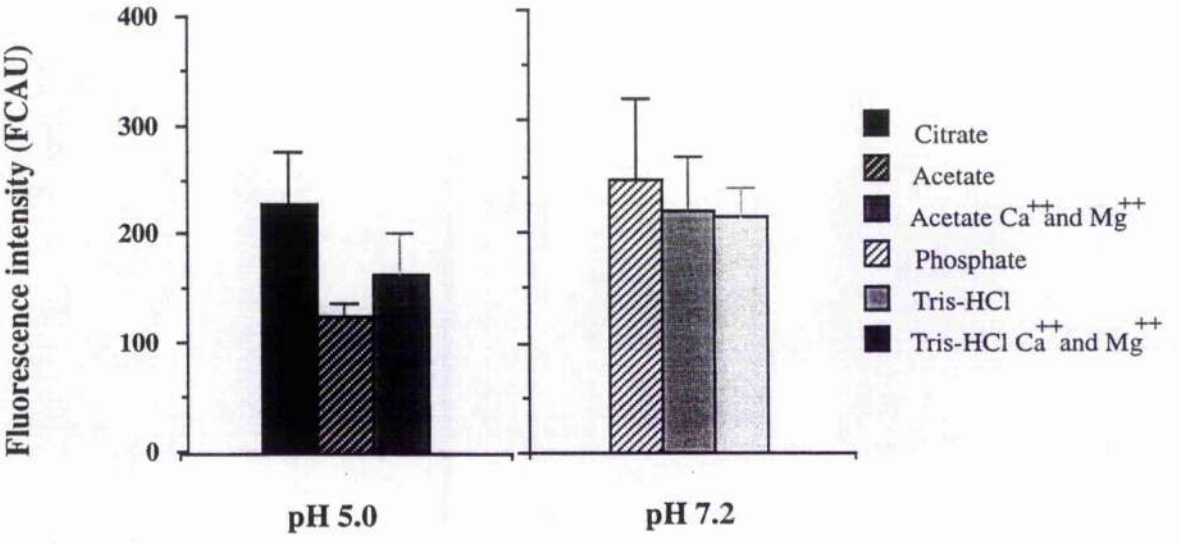


Figure 48. Flow cytometry of *C. albicans* : Summary bar charts to show binding of the fucose probe by the hyphal-form cells in different buffers at pH 5.0 and 7.2 with or without Ca^{++} and Mg^{++} Vertical lines represent SEM

A. Percentage of the cells labelled with the probe within marker limits



B. Fluorescence intensity of the cells labelled with the probewithin marker limits



For clarity of presentation, fluorescence intensity of the background readings in the set marker which were very low, are omitted.

Figure 49. Flow cytometry of *C. albicans*: Effect of EDTA on binding of the fucose probe by the hyphal form cells. The diagrams show single parameter histograms. The grey line represents unlabelled cells and the black area the cells labelled with the probe

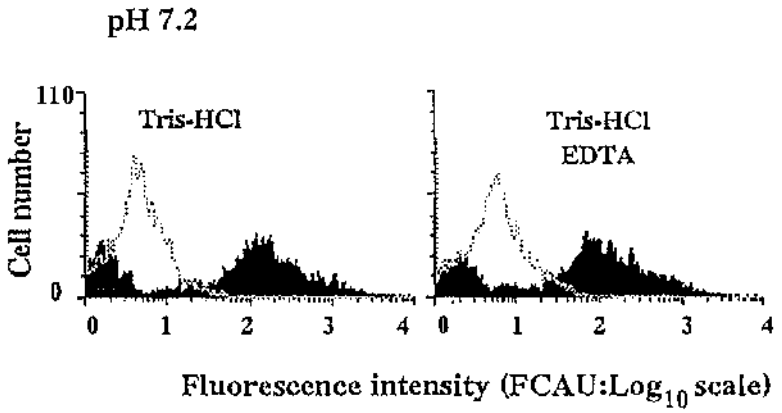
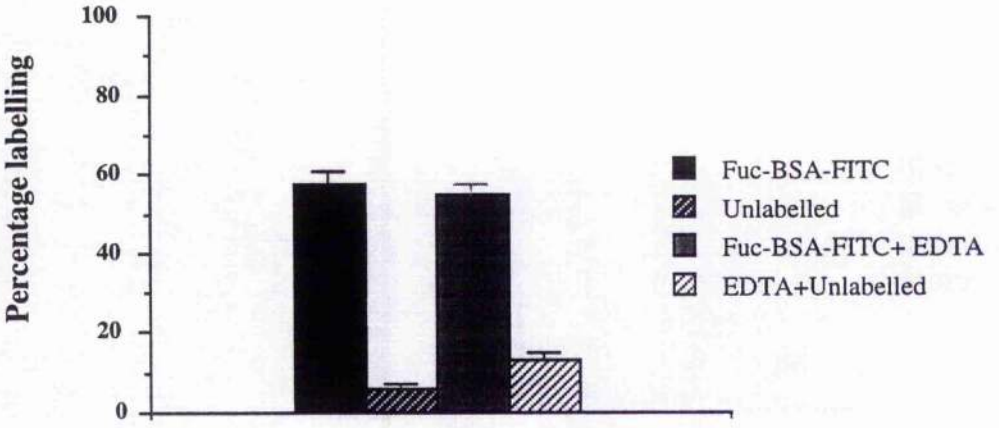


Figure 50. Flow cytometry of *C. albicans* : Summary bar charts to show effect of EDTA on binding of the fucose probe by the hyphal-form cells. Vertical lines represent SEM

A. Percentage of the cells labelled with the probe, or unlabelled, within marker limits



B. Fluorescence intensity of the cells labelled with the probe, or unlabelled, within marker limits

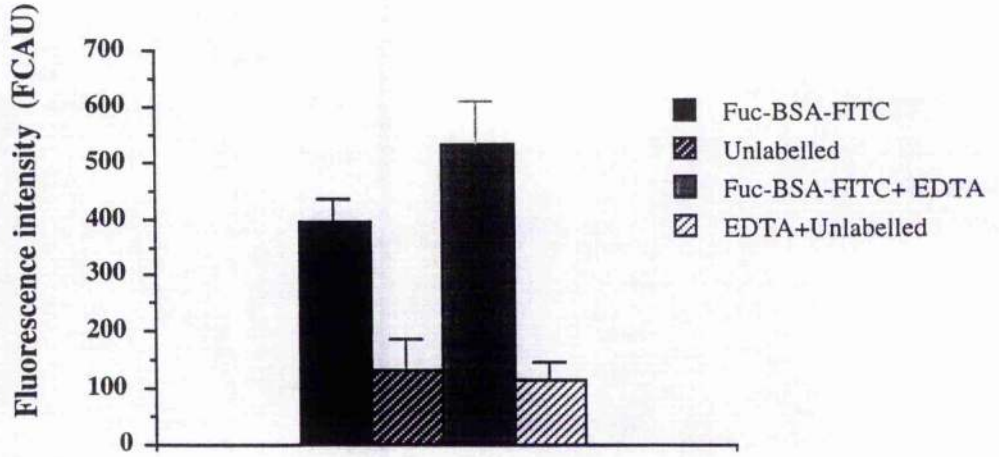


Figure 51. Flow cytometry of *C. albicans*: Effect of different temperatures on binding of the fucose probe by the hyphal-form cells. The diagrams show single parameter histograms. The grey line represents unlabelled cells and the black area the cells labelled with the probe

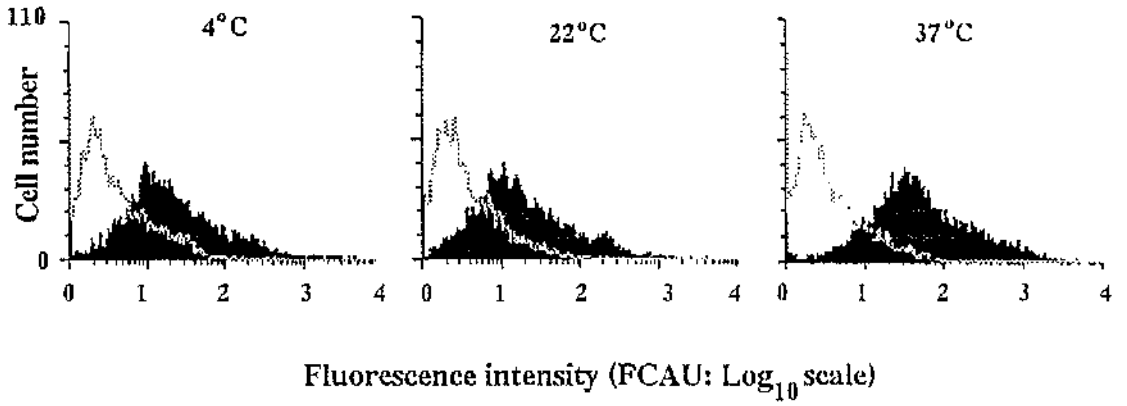
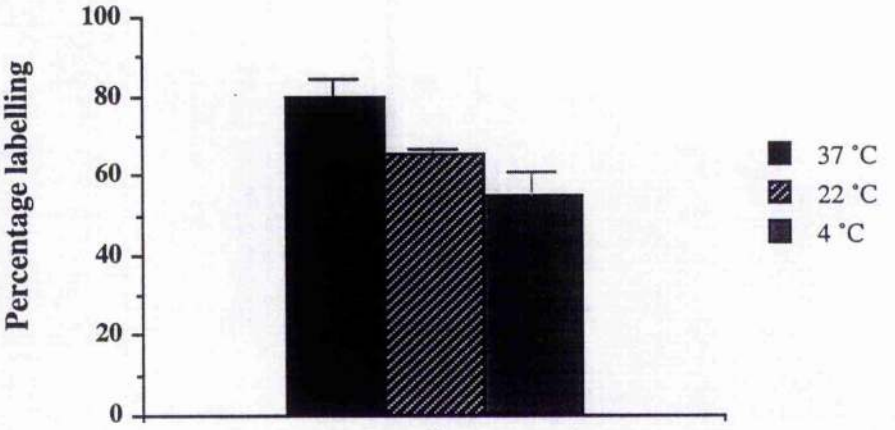
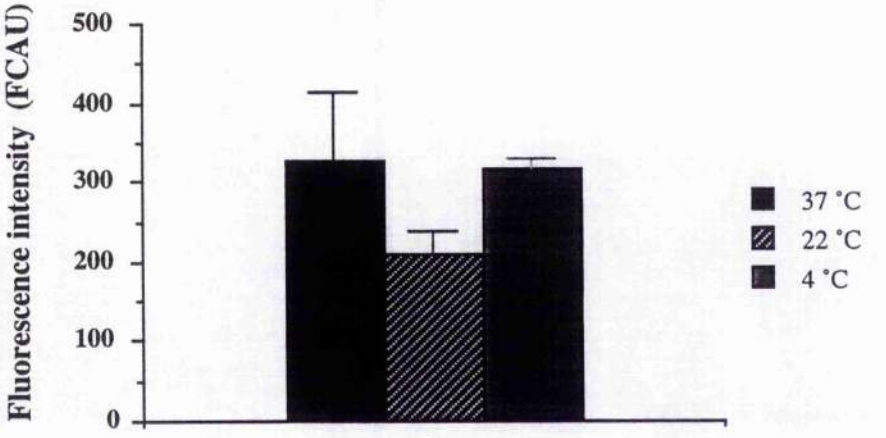


Figure 52. Flow cytometry of *C. albicans* : Summary bar charts to show the effect of different temperatures on binding of the fucose probe by the hyphal-form cells. Vertical lines represent SEM

A. Percentage of the cells labelled with the probe within marker limits



B. Fluorescence intensity of the cells labelled with the probe within marker limits



For clarity of presentation, fluorescence intensity of the background readings in the set marker which were very low, are omitted.

Figure 53. Flow cytometry of *C. albicans*: The binding of the fucose, lactose and BSA probes by the hyphal-form cells. The diagrams show single parameter histograms. The grey line represents unlabelled cells and the black area the cells labelled with one of the probes

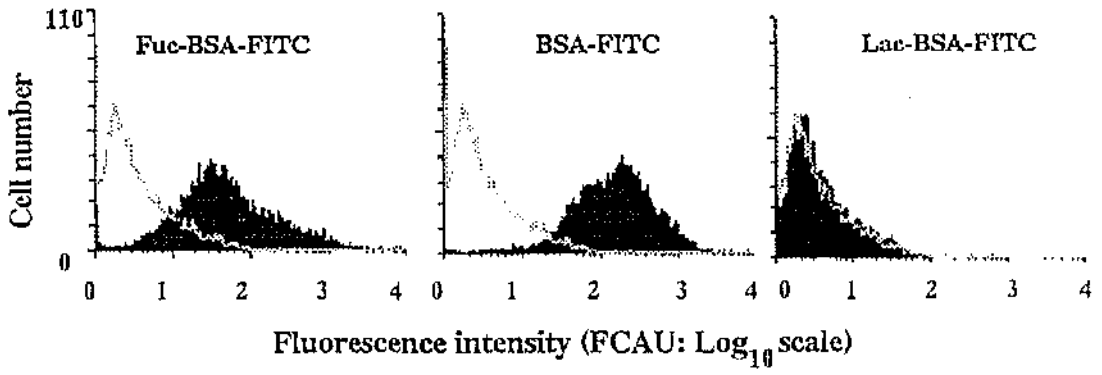
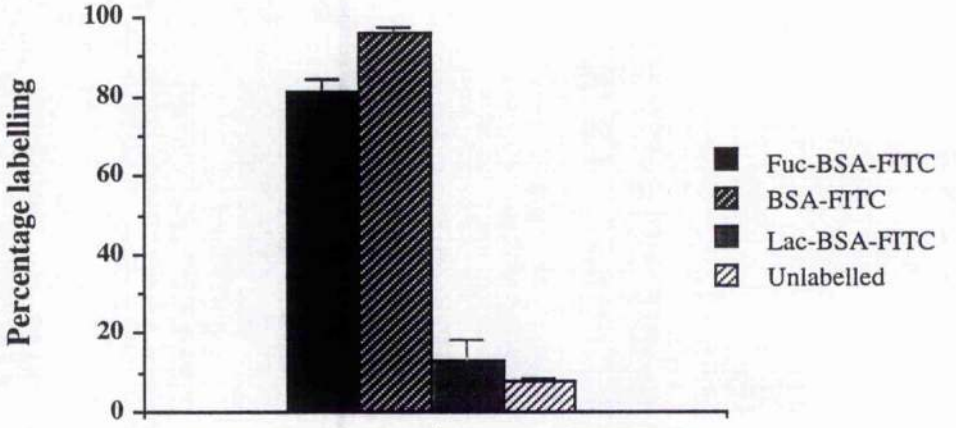


Figure 54. Flow cytometry of *C. albicans* : Summary bar charts to show the binding of fucose, lactose and BSA probes by the hyphal-form cells. Vertical lines represent SEM

A. Percentage of the cells labelled with the probes, or unlabelled, within marker limits



B. Fluorescence intensity of the cells labelled with the probes, or unlabelled within marker limits

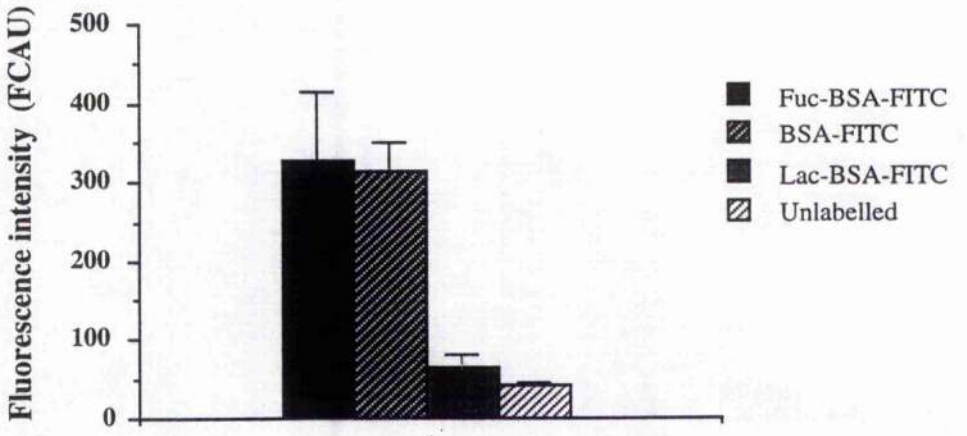


Figure 55. Flow cytometry of *C. albicans* : Inhibition binding of the fucose probe (FITC-labelled) by unlabelled Fuc-BSA, Gal-BSA L-Fucose and D-Galactose. The diagrams show single parameter histograms. The grey line represents unlabelled cells and the black area the cells labelled with the probe

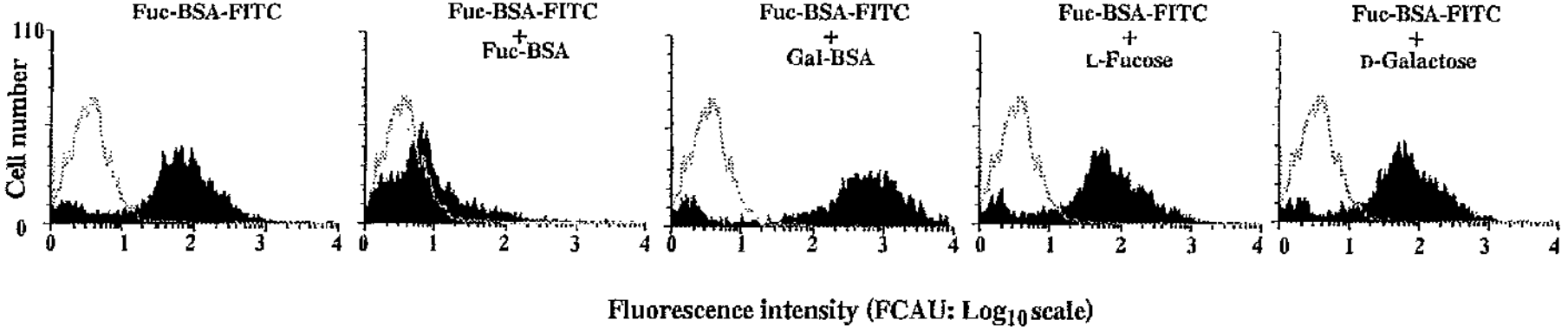
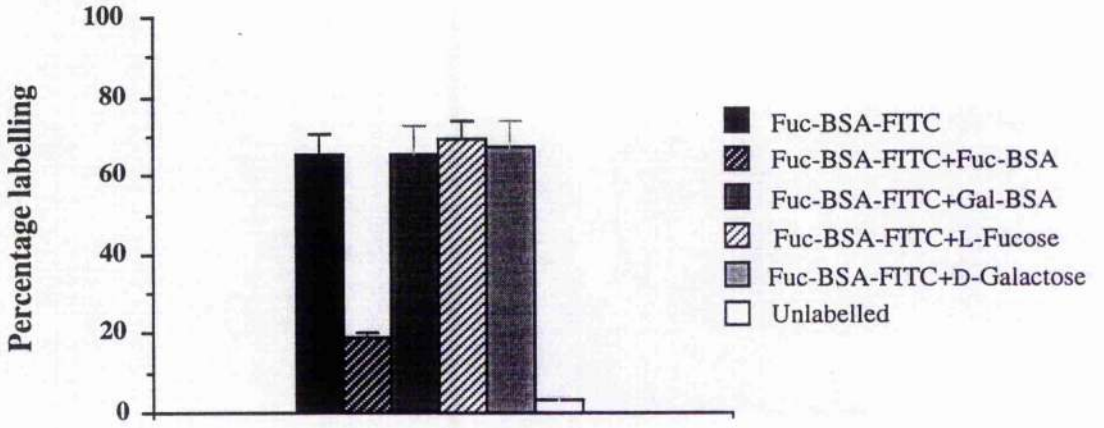


Figure 56. Flow cytometry of *C. albicans* : Summary bar charts to show inhibition of binding of the fucose probe (FITC-labelled) by unlabelled Fuc-BSA, Gal-BSA, L-Fucose and D-Galactose. Vertical lines represent SEM

A. Percentage of the cells labelled with the fucose probe, or unlabelled, within marker limits



B. Fluorescence intensity of the cells labelled with the fucose probe, or unlabelled, within marker limits

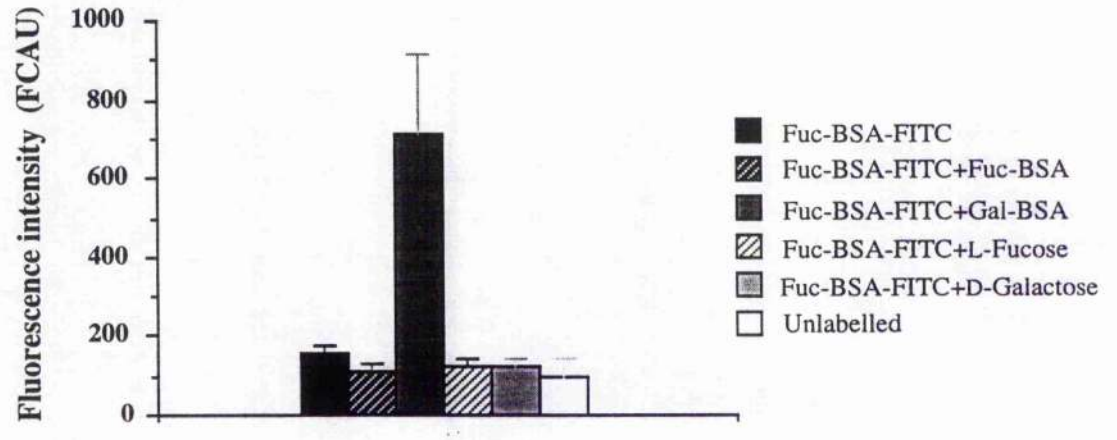


Figure 57. Flow cytometry of *C. albicans* : Growth of wild type cells (strain MEN) and its germ tube-deficient mutant (strain MM2002) in 199 Medium at pH 6.7 and 37 °C, showing distribution of cell size and density during germ tube production. The diagrams are dual parameter contour plots

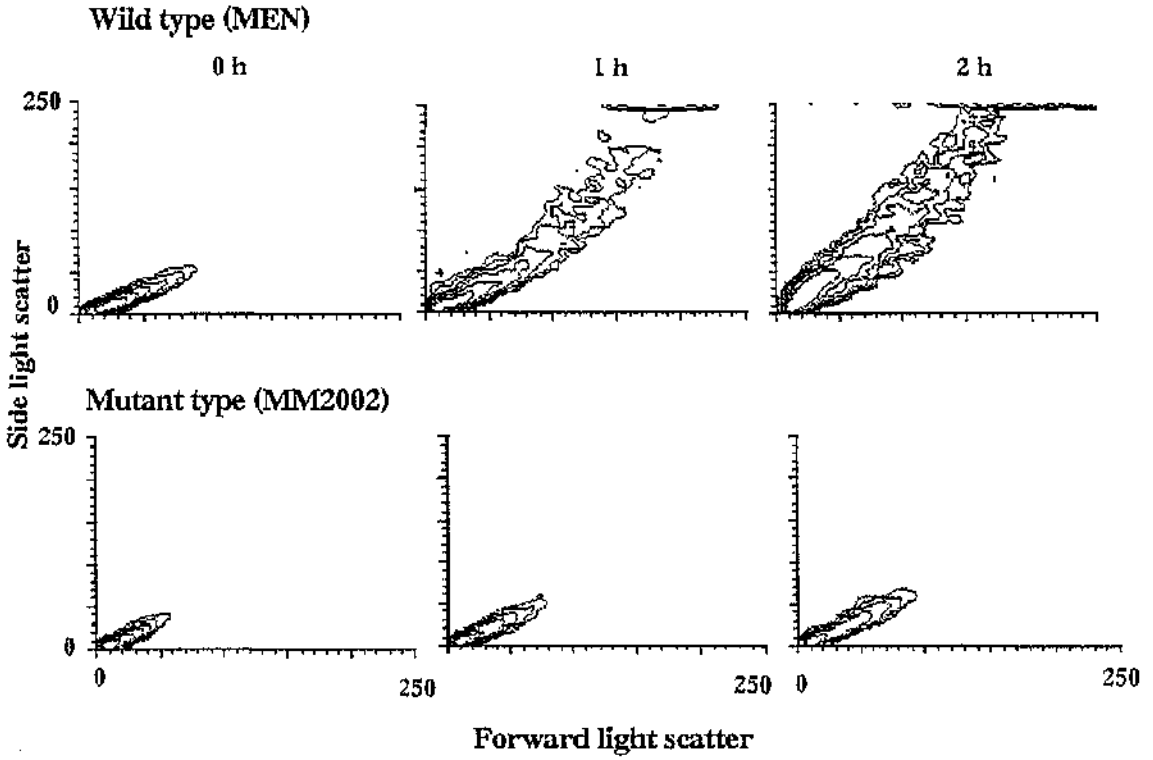


Figure 58. Flow cytometry of *C. albicans*: Binding of the fucose probe by wild type cells (strain MEN) and its germ tube-deficient mutant (strain MM2002) during germ-tube production. The diagrams show single paramater histograms. The grey line represents unlabelled cells and the black area the cells labelled with the probe

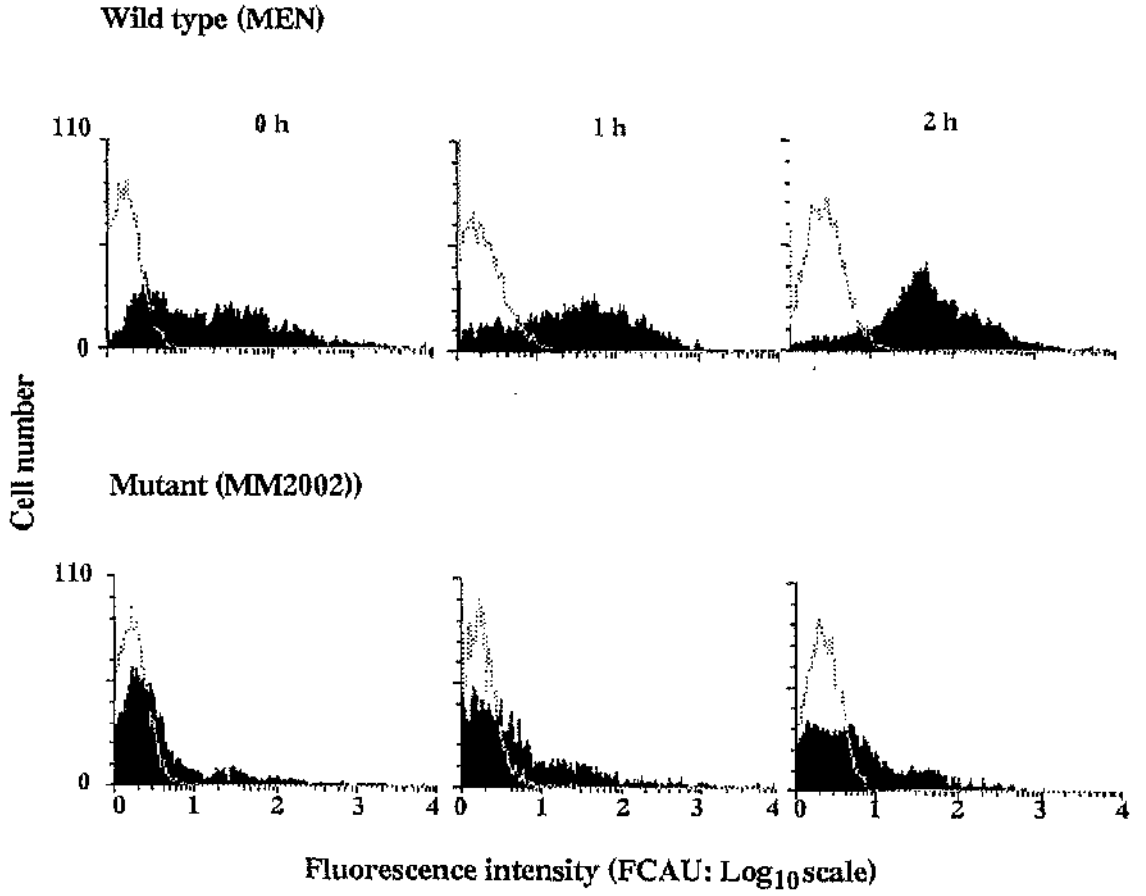
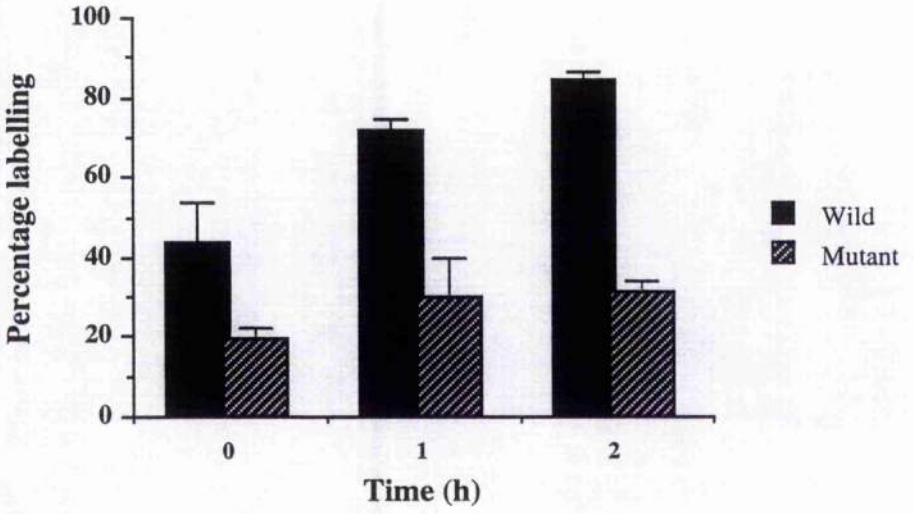
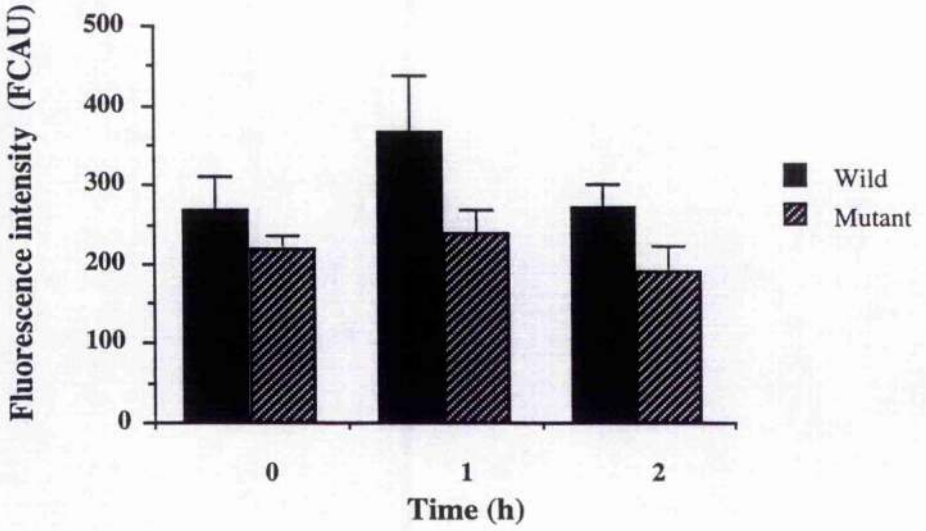


Figure 59. Flow cytometry of *C. albicans* : Summary bar charts to show binding of the fucose probe by wild type (strain MEN) and its germ tube-deficient mutant (strain MM2002) during favouring conditions germ tube production. Vertical lines represent SEM

A. Percentage of the cells labelled with the probe within marker limits



B. Fluorescence intensity of the cells labelled with the probe within marker limits



For clarity of presentation, fluorescence intensity of the background readings in the set marker which were very low, are omitted.

4.4. FIBRONECTIN ADHESINS OF *CANDIDA ALBICANS*

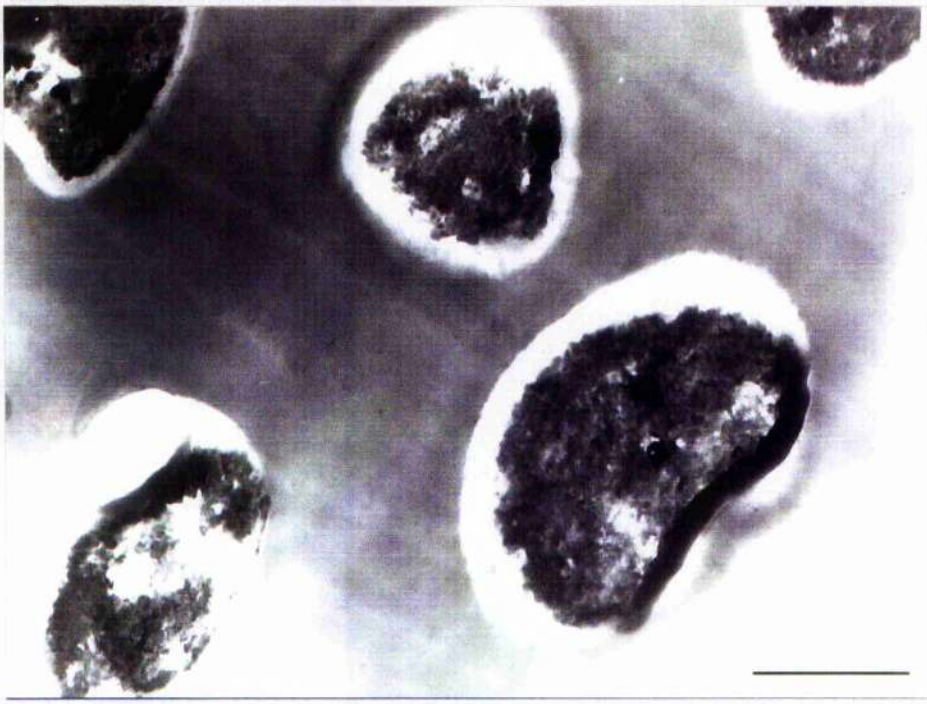
Binding of fibronectin to the yeast and hyphal-form *C. albicans* was explored using 20 nm colloidal gold particles coated with fibronectin (Au_{20}-Fn). This technique was used by Pesciotta Peter and Moster (1987) to show fibronectin receptors on human fibroblasts. This reagent was added to yeast cells of *C. albicans* ($2x10^8$ cells ml^{-1}), which had been grown on Yeast Nitrogen Base Medium containing Galactose (YNBGal) and Modified Lee Medium without amino acids (MLMwAA). Figure 60A and B are transmission electron micrographs of the yeast-forms cells grown in both media which do not show any fibronectin coated gold particles on the their surface.

In a similar fashion, *C. albicans* grown in 199 Medium (pH 6.7) at 37°C for 1 h and 2 h under conditions for production of germ tubes were labelled with Au_{20}-Fn. There were clearly visible Au_{20}-Fn particles on the germ tube portion of hyphal-form cells (1 h) and few if any on the yeast-cell portion (Figure 60C). The particles were shown by arrows in the figure. Figure 60D shows that the hyphal-form cells (2 h) labelled with Au_{20}-Fn had gold particles on the germ tube portion.

Control experiments with PEG-coated gold particles showed that Au_{20}-PEG had little affiniy for the germ tube portion of hyphal-form cells (Figure 61).

Figure 60. Transmission electron microscopy of *C. albicans* : Micrographs of the cells labelled with fibronectin coated-gold particules

A. The yeast-form cells were grown in YNBGal 12000 x. Bar represents 1 µm

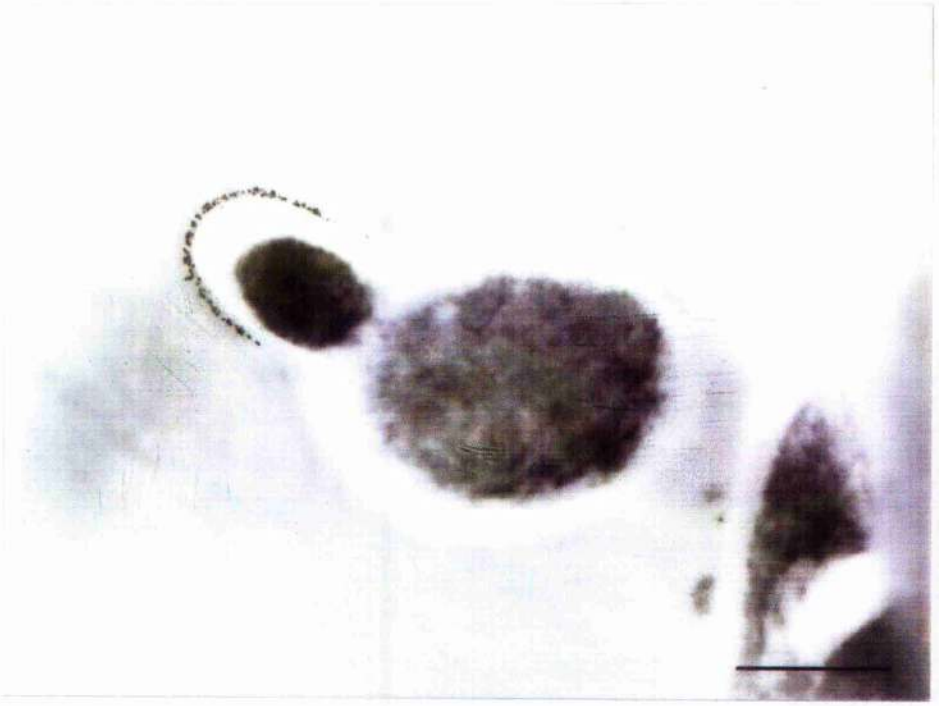


B. The yeast-form cells were grown in MLMwAA 12000 x. Bar represents 1 µm

Arrows indicate the cells labelled with fibronectin coated-gold particles.

C. The hyphal- form cells were grown in 199 Medium for 1 h at 37°C (a,b)

a. 12000 x. Bar represents 1 μm



b. 7.000 x. Bar represents 1 μm

D. The hyphal- form cells were grown in 199 Medium for 2 h at 37°C 4400 x. Bar represents 2 μm

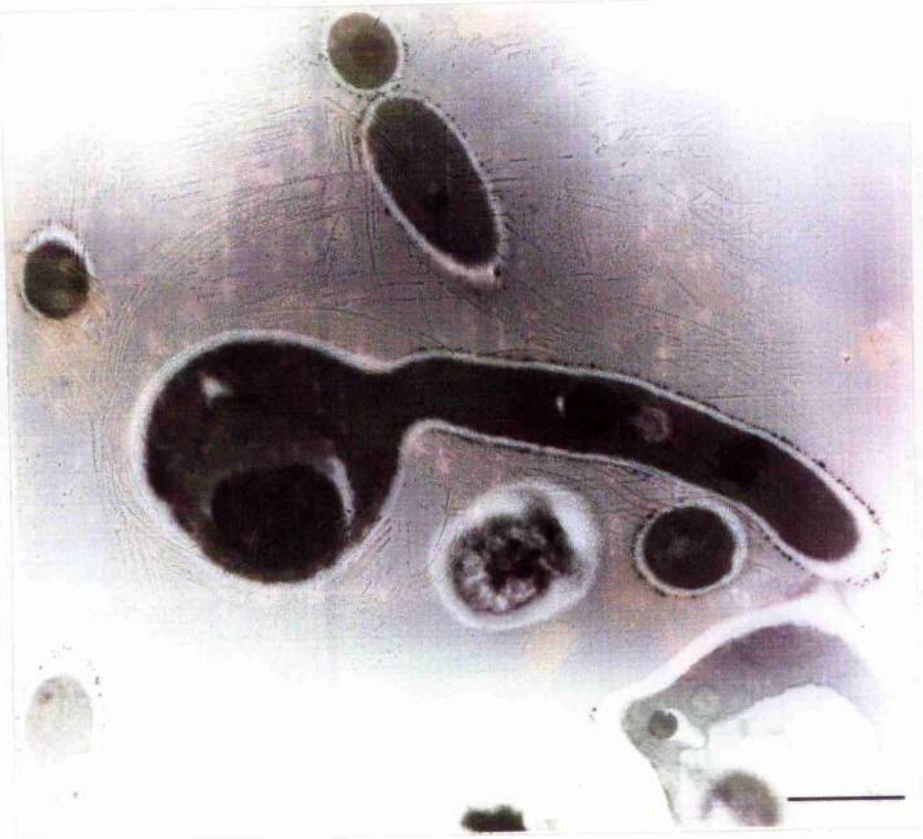
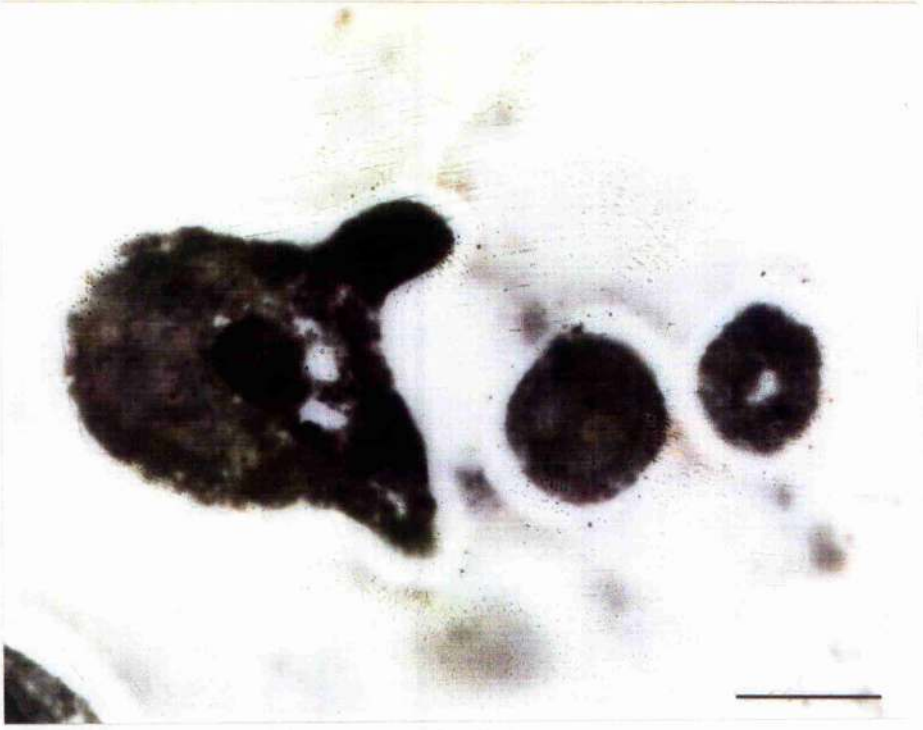


Figure 61. Transmission electron microscopy of *C. albicans* : Micrographs of the hyphal-form cells labelled with PEG-coated gold particules 12000 x. Bar represents 1 µm



5. DISCUSSION

5.1. GENERAL OBSERVATIONS

Adherence of *C. albicans* to host cells is considered a critical step in the pathogenesis of candidiasis (Odds, 1986). The conversion of *C. albicans* from the commensal to the pathogenic state is associated with its ability to make the dimorphic transition from the yeast cell to the hyphal form (Hill and Gebhardt, 1956; Kimura and Pearsall, 1980), although the evidence in support of this is equivocal (Cutler, 1991 and Odds, 1988). This phenomenon of dimorphism must involve reorganization of the cell wall at the molecular level, a background to the studies described in this thesis.

In this study, the morphological development of *C. albicans* cells uses the following terminology: The yeast-form cell is the unicellular form of the fungus which propagates itself by budding. Under certain environmental conditions the yeast-form cell produces a cylindrical structure - the germ tube which is the initial morphological form of hyphae (Cassone *et al.*, 1973). The two parts are then described as the germ-tube portion and the yeast-cell portion. The whole structure is referred to as a hyphal-form cell.

5.2. ADHESION OF *C. ALBICANS* GERM TUBES TO PETRI DISHES

5.2.1. Germ Tube Formation by *C. albicans*

In *C. albicans* germ tube production can occur rapidly in serum, serum substitutes and other natural and synthetic media. The production by *C. albicans* of germ tubes in serum is important for species identification (Taschdjian *et al.*, 1960). At the start of this research there appeared to be no generally accepted procedure for rapid and complete production of germ tubes in a defined medium. For this reason some effort was devoted in the early stages to finding a suitable medium, and three defined media

were tested. Two of them, 199 Medium and Glucose-Glycine Medium (GG), gave approximately 90 % germ tubes in 140 min and were equally satisfactory. With both of them, an essential part of the overall procedure was 36 h starvation beforehand in Modified Lee Medium without aminoacids (MLMwAA), as reported by Tronchin *et al.* (1988).

Complex synthetic media such as 199 Medium which were originally designed for tissue culture work have been widely used for production of hyphal-form *C. albicans* (Dabrowa *et al.*, 1968, 1970; Rotrosen *et al.* 1985, Tronchin *et al.*, 1988). The principal nitrogen source is various aminoacids and the main carbon source is glucose. The mixture of aminoacids in 199 Medium may therefore stimulate hypha formation by processes similar to those of serum.

Muerkoester *et al.* (1979) compared GG medium with five other media, namely neopeptone-starch broth, Lee medium, non-sterile pooled human serum, sterilized pooled serum and Sabouraud's dextrose broth. They showed that GG Medium gave 70% germ tube production within 3.5 h at 37°C, which was a higher percentage and more rapid production of these structures than with the other media tested. Mr A.I. Altabet from our laboratory (personal communication) also used these six media and observed that 90% hyphal-form cells were produced by *C. albicans* in GG Medium at 37°C for 3.5 h. Thus although there is good evidence for similar germ-tube production in 199 Medium and GG Medium, the former was mostly used in the present work. This was mainly because of its preferred use by other investigators (Tronchin *et al*, 1988; Tronchin *et al*, 1989; Bouchara *et al.*, 1990).

Among the less suitable media for germ-tube production was Sucrose-Gelatine Medium (SG) which gave only about 15 % germ-tube production after previous growth of the organisms in YNB containing glucose and L-asparagine, as described by Mayer *et al.* (1992). These authors prepared yeast cells from three consecutive passages in the YNB containing glucose and L-asparagine, with final resuspension in SG. After the 1 h incubation at 37°C used by these authors, the percentage of germ tubes was not

reported. But it should be remembered that a low percentage of germ-tube production by *C. albicans* could be due to inappropriate preincubating conditions.

Although the production of germ tubes by yeast cells of *C. albicans* in serum is a useful rapid test for identification since it gives results within 2-3 h, the chemical complexity of serum is probably a disadvantage for basic studies of components on the surface of the germ-tube and yeast-cell portions of the cell, as described below.

5.2.2. Production and Detection of Mannoprotein Adhesins on Plastic Petri Dishes

The adherence of yeast-form *C. albicans* to inert surface such as acrylic was observed by Samaranayake and MacFarlane (1980) and by McCourtie and Douglas (1981). When the yeast cells were grown to stationary phase in media containing a high concentration of certain sugars, *C. albicans* produced an additional surface layer which was probably responsible for this enhanced adherence (McCourtie and Douglas, 1981). The attachment of the organism to plastic surfaces has been well demonstrated and may be important in infections involving dentures, prosthetic cardiac valves and intravascular and urinary catheters (Rotsosen *et al.*, 1986). These devices may thus promote *C. albicans* invasion of the human host (Martin *et al.*, 1984; Sobel *et al.*, 1984, and Klotz *et al.*, 1985).

Previous research has shown that both yeast cell and hyphal-form *C. albicans* can adhere to host surfaces. But it was suggested that the hyphal-form was more adherent to epithelial cells than was the yeast form (Kimura and Pearsall, 1980). There was a strong correlation between germ-tube production and adherence to plastic of *C. albicans* reported by Tronchin *et al.* (1988). In the present research, production of hyphal-form *C. albicans* in 199 Medium at pH 6.7 was done in polystyrene petri dishes. After removing the cells with a plastic scraper from the dishes, the mannoprotein adhesins were detected on the plastic surface by use of Con A-coated latex beads, the mannoprotein having a specific capacity to bind concanavalin A. This binding occurred on the plastic surface along the impressions left by the germ tubes

which had previously been removed with the plastic scraper. This result indicated that germ-tube formation may cause chemical changes in the cell wall mannoprotein adhesins and enhance adherence to plastic. The same result had been obtained by Tronchin *et al.* (1988) after removing the cells with a rubber policeman from the dishes. Although this procedure had been removed in our previous experiment, some of the cells were retained on the dishes. Confirmation of Con A-adhesins on the germ tube cell wall was obtained by labelling of the cells with Con A-gold particles under electron microscopy by Tronchin *et al.* (1988). Although these authors did not report protein and carbohydrate content of mannoprotein adhesins or make test for inhibitory activity, they purified 4 different proteins after dithiothreitol and iodoacetamide treatment of the washed plastic petri dishes. My work thus confirmed that the mannoprotein adhesins remained on the plastic, even after the organisms had been removed with a plastic scraper. Moreover, control petri dishes with uncoated-latex beads and Con-A treated latex beads, together with α–methyl-D-mannopyranoside, were free of beads. Control petri dishes in which only yeast cells were produced contained few Con A treated-latex beads, showing that yeast-form cells adhered only weakly to the plastic under the same conditions (except temperature) used with germ tubes.

5.2.3. Inhibitory Activity of Mannoprotein Adhesins

Although mannoprotein adhesins (MPA) were observed on the plastic surface by Tronchin *et al.* (1988), these authors did not test the possible inhibitory activity of hyphal form MPA towards adhesion of yeast-form *C. albicans* on human buccal epithelial cells (BEC). Other investigators have isolated extracellular polymeric material (EP) of yeast-form *C. albicans* from culture supernates and shown blockage of adherence by pretreating the epithelial cells before use in adherence assays (McCourtie and Douglas, 1985; Critchley and Douglas, 1987a). In these experiments, EP (crude adhesin) was isolated from *C. albicans* 2346 grown in defined medium containing a high concentration of galactose (Critchley and Douglas, 1987a). Recently, Tosh and

Douglas (1992) purified a fucose-binding protein fragment by affinity adsorption with the trisaccharide determinant of the H (type 2) blood group antigen which terminates in a residue of L- fucose. The purified adhesin inhibited yeast adhesion to buccal epithelial cells. Adhesion inhibition was observed up to a maximum of 80 % at an adhesin concentration of 10 μg ml^{-1}. These observations thus implicated the fucose-binding protein as the major adhesin of yeast cells of *C. albicans*.

In the present study, mannoprotein adhesins of germ tubes of *C. albicans* from the petri dishes (1 mg ml^{-1} of protein) were used to pretreat epithelial cells prior to their exposure in an adherence assay. These pretreated epithelial cells were then mixed with yeast-form *C. albicans* to test for inhibitory activity of the mannoprotein adhesins, which a had protein: carbohydrate ratio of 3 : 1. The outcome was that the crude germ-tube mannoprotein adhesins, at the concentrations tested, were not able to block yeast adhesion to the epithelial cells. Thus the mannoprotein adhesins of the hyphal form cells were not identical to those from the yeast cells.

Germ-tube mannoprotein adhesins have been used for the identification of laminin, fibrinogen and C3d receptors (Bouchara *et al.*, 1990, Tronchin *et al.*, 1989) on SDS-PAGE followed by blotting on nitrocellulose membranes. The active components were molecules of 60 and 68 kDa and had multiple activities.

Critchley and Douglas (1987a) reported that treatment of their crude extracellular material from yeast-cell supernates with dithiothreitol abolished its inhibitory effect for adhesion of *C. albicans* to epithelial cells. In the present work, dithiothreitol was used to remove the mannoprotein adhesin from the petri dishes and the disulphide bonds thereby broken may have been involved in maintaining the integrity of the protein. Perhaps distraction of disulphide bridges caused the material to be inactive in the adhesion assay. Also, the adhesin molecules might be smaller than 12,000 Molecular weight (MW) and during dialysis, might be lost.

5.3. LECTIN-LIKE ADHESINS OF *C. ALBICANS*

5.3.1. Identification of Lectin-Like Adhesins

C. albicans is a dimorphic fungus where, as noted above, the yeast form is generally associated with a commensal relationship while the additional presence of the hyphal form is associated with infection (Calderone, 1993). A number of proteins are reported to be preferentially expressed on the surface of the germ-tube portion of hyphal-form *C. albicans* (Bouali *et al.*, 1987; Bouchara *et al.*, 1990; Martinez, *et al.*, 1994).

On bacterial surfaces Ofek *et al.* (1977) suggested that proteins with lectin-like properties could serve as adhesins for binding the organisms to host cells. Thus *E. coli* with type 1 fimbriae was specific for mannose-containing receptors. Lectin-like adhesin molecules were identified on yeast-form *C. albicans* by Critchley and Douglas (1987b) and by Tosh and Douglas (1992) in this laboratory. Such molecules were not observed on hyphal-form *C. albicans*. Progress in this area was greatly helped by availability of neoglycoproteins (Sigma). These consist of carrier protein such as albumin, to which chosen sugars are covalently bonded, together with various cytochemical markers, notably fluorescein, colloidal gold, ^{125}I or latex minibeads. This use of carbohydrate-protein labelled with FITC conjugates to identify carbohydrate-binding proteins was introduced by Monsigny *et al.* (1984). The neoglycoproteins were used by Depierreux *et al.* (1991) for characterization of *Agrobacterium tumefaciens* lectins. Masy *et al.* (1991) employed them for visualisation and quantitation of the lectin-like receptors involved in the flocculation of the yeast *Saccharomyces cerevisiae*. Surface sugar-binding proteins of bovine spermatozoa were identified by Sinowatz *et al.* (1988).

In using these reagents, it is essential to make control experiments with neoglycoproteins containing a range of different sugars, and also a sugar-free carrier protein with the FITC or other cytochemical marker. Specificity of reaction may be explored by inhibition studies with high concentrations of the free sugar. The following sections describe the use of neoglycoproteins to investigate the lectin-like receptors on hyphal-form *C. albicans* by a variety of technologies.

5.3.1.1. Fluorescence microscopy

Fluorescein-labelled neoglycoproteins were shown to be suitable for visualisation of membrane lectins of mouse and human lymphocytes, and Lewis lung carcinoma cells under the fluorescence microscope (citied by Monsigny *et al.*, 1984). In the present work, fluorescence microscopy was a useful qualitative tool for observing the distribution of the fucose probe labelled with FITC on hyphal-form *C. albicans*. Of the five neoglycoproteins used, by far the most active was fucose-BSA-FITC. However, the other neoglycoproteins (labelled with mannose, glucose, galactose and lactose) which were apparently less well bound, might have been less visible because of stronger quenching under blue-light illumination. In *C. albicans* grown in YNBGal and Modified Lee Medium without aminoacids, the micrographs showed the fucose probe as small spots on the yeast-form cells. On hyphal-form *C. albicans* grown in the 199 Medium for 2.5 h, the fucose probe bound to both portions of the cells, but there was more probe on the germ tube portion. This indicated that the germ tube portion is the newly-produced portion and might present more lectin-like adhesins which were specific for the fucose probe than the yeast portion. Hyphal-form cells grown in 199 Medium for 4 h showed a similar distribution to the cells grown in the same medium for 2.5 h. The use of Glucose-Glycine Medium with the same strain for 4 h gave labelling similar to that of cells grown in 199 Medium for 4 h.

The length of the hyphal-form cells might be a disadvantage for focusing of the whole cell under the blue light for fluorescence microscopy. This difficulty could be reduced by serial optical sectioning by fluorescence confocal microscopy so as to provide high-resolution three-dimensional images of the distribution of labels on the cell wall (Shotton and White, 1989). Distribution of fibrinogen-binding protein on hyphal form *C. albicans* has been examined by fluorescence confocal microscopy (Martinez *et al.* 1994) and more detail about the heterogeneous distribution in the cell wall thus provided. It would clearly be of interest to apply this technique to determining the distribution of lectin-like adhesins.

Photography of labelled cells is associated with an optical quenching problem (i.e. chemical destruction of fluorescein under the intense blue light) which was effected by exposure time when yeast and hyphal-form *C. albicans* labelled with fucose probe were photographed. Although the mannose and the glucose probes could be seen by eye to bind less than the fucose probe on the yeast and hyphal form cells, micrographs could not be obtained because of the problem.

5.3.1.2. Image analysis

Image analysis has been used to describe the "morphology index" of yeast and hyphal-form *C. albicans* (Merson-Davies and Odds, 1989) and to explore the attachment of the yeast cells to host tissue (Riesselman *et al.*, 1991). In the present study, the basic fluorescence image analysis system consisted of a microscope with blue light illumination, excitation and emission monochromators, a video camera, and a computer with digitizing peripherals. The output was an electronic video image.

Visualization of distribution pattern of membrane-associated calcium and Calmodulin during pollen hydration and germination, and tip growth in *Nicotiana tabacum* have been documented using image analysis by Tirlapur and Cresti (1992).

For observation of lectin-like adhesins on hyphal-form *C. albicans*, aliquots of appropriately-grown cells were labelled with the five neoglycoprotein probes containing fluorescein. The images under both bright field and blue light were copied to the hard disk and printed on special paper. Thus the image of an individual cell under bright field could be compared with its fluorescence counterpart so as to identify the sites and distribution of probe-binding. To minimize the quenching problem the video images were captured and stored as rapidly as possible.

Printer papers from the image analyser demonstrated that the fucose probe bound to both yeast and germ tube portions of hyphal-form *C. albicans*. Fluorescence intensity of mannose and other probes was more detectable by image analysis than the less sensitive fluorescence microscopy. The use of the very low-light TV camera permitted observation of very low intensities from the probe-labelled preparations

which would have been undetected even by exposures of many minutes with fast photographic film. Contour maps and perspective plots of blue-light images additionally gave details about the fluorescence intensity of all five probes on the cells. Image analysis was used not only for visualization of the probes on the cells, but also for measurement of fluorescence intensity of the probes for semi-quantitation. The printer papers of the computer output of the hyphal-form cells labelled with the fucose probe showed similar patterns to the photographs obtained by fluorescence microscopy.

5.3.2. Quantitation of Lectin-Like Adhesins of *C. albicans*

5.3.2.1. Spectrofluorimetry

In the present study, spectrofluorimetry was used to quantitate the binding of the neoglycoproteins to yeast and hyphal-form *C. albicans* GDH 2346. Probes of this type had previously been used by Masy *et al.* (1991) to characterize lectin-like receptors at the surface of *Saccharomyces cerevisiae*, and by Depierreux *et al.* (1991) to characterize the lectins of *Agrobacterium tumefaciens*. Both groups used spectrofluorimetry on supernates after the samples had been washed with a flocculation inhibitor which was either EDTA or mannose to induce release of the probes bound on the cells (Masy *et al.*, 1991). Alternatively, the cells were digested with pronase B at pH 7.4 to release fluoresceinylated peptides from the cell envelope (Depierreux *et al.*, 1991).

In my work, binding of neoglycoproteins on hyphal-form *C. albicans* was dependent on pH. All five probes were affected. Also, there was lower fluorescence intensity at pH 5.0 than at pH 7.2. However, at both pHs the fluorescence intensities of the probes at pH 5.0 and 7.2 were increased after degradation with pronase. These observations were in agreement with these of Midoux *et al.* (1987) who also found that the fluorescence intensity of pronase-treated fluorescein-labelled neoglycoproteins increased linearly as the degree of labelling increased. The experiment with the fucose

probe over the pH range from 3.0 to 7.0 showed with both yeast and hyphae that binding was maximum at pH 5.0. At lower pH values (3.0 - 4.0), the probe was precipitated. The mannose probe also showed a maximum at pH 5.0 but which was poorly-defined. The glucose probe seemed to bind less than the mannose probe, and the lactose probe apparently failed to bind.

A saturation curve of the fucose probe was done by with the yeast and hyphal-form cells and showed that the fucose binding sites were becoming saturated at a probe concentration of 100 µl ml^{-1}. This saturation indicated that binding sites were limited in their number. Calculation indicated that each hyphal-form cell of *C. albicans* possessed about 2 $x10^7$ fucose-probe-binding sites, although the number may be higher. After pronase degradation of the probe bound on the cells, the fluorescence intensity of the released probe introduced an uncertainty in the above calculation. Despite this limitation, neoglycoproteins are useful tools for characterization of membrane lectins although certain problems of quantitation arise in using spectrofluriometry.

To compare lectin-like adhesins on the hyphal-form and yeast-form *C. albicans*, germ-tube production was investigated at different pHs and temperatures in 199 Medium, before labelling with the fucose probe. It was observed that the cells in the 199 Medium at pH 6.7, at 37°C, and which had produced germ tubes after 1 h, grew further during subsequent incubation. During this process, binding of the fucose probe increased. The fucose probe bound less with cells prepared under other conditions viz: 199 Medium at pH 6.7 at 22°C, and the same at pH 4.0 at 37°C, neither of which was suitable for germ tube production.

5.3.2.2. Image Analysis

The technique of computer-assisted image analysis was used for visualization and semi-quantitation of the fucose probe on yeast and germ tube portions of hyphal-form cells. In this method, an image of the field observed by microscopy allowed selection of

individual cells for analysis. It also allowed precise localisation and semi-quantitation of optical signals emitted by each point of observed fields on the cells.

In this study, semi-quantitation of fluorescence intensity of the cells, and parts of them, was done with two computer programs which measured on large and small scales. Thus after the cells had been labelled with the fucose probe, the fluorescence intensity of each portion of the cells in the big square (100x100 pixels) was measured. The intensity of fluorescence in the cultures of rat epitenon fibroblasts labelled with anti-fibronectin antibodies was measured by the same image analysis program (Wójciak and Crossan, 1994). But, this revealed no differences between yeast and germ tube portions in their fluorescence intensities. Fluorescence intensity of the cell portions was decreased because of dark area around the cells portions in the big squares. A significant technical problem in this work was the quenching of fluorescence under the intense illumination, although this was minimized by careful control of exposure time.

With the small squares, 10x10 pixels (less than 1μm) of the cell was divided up from the tip of the germ tube back to the different areas on the yeast cell portion. There was clear evidence that the germ-tube areas had more fucose probe than the yeast-cell areas. However, fluorescence intensities were highest in the middle portion of the germ tubes. But there could be a geometrical difficulty because of the alignment of the surfaces in tip regions of the germ-tube portions. These observations were made on many hyphal form *C. albicans* and are believe to indicated that the germ-tube portions have high level of binding for the fucose probe.

Adhesin molecules may be heavily expressed on hyphal and pseudohyphal form cells than yeast forms (Cutler, 1991). The association of fucose-specific lectin-like adhesin on hyphae does not mean yeast form are less virulent because at least the adhesin molecules are expressed by both forms. If would be change to measure the fucose probe on buds of yeast forms, because new synthesised part of the cell could give higher fluorescence of the fucose probe.

5.3.2.3. Flow cytometry

Flow cytometry (FCM) allows analysis of a microorganism suspension, cell by cell, through quantification of optical signals and rapid analysis. Several hundred cells per second is possible. The technique had already been used with *C. albicans* in the following areas: to detect surface iCb3 receptors (Gilmore *et al*, 1988) ; to observe the relationship between fibrinogen binding and germ-tube formation (Lynch *et al.*, 1993); to monitor chitin synthesis (Hector *et al,* 1990); to measure susceptibility to antifungals (Pore, 1990; Green *et al*, 1994); to monitor attachment to buccal epithelial cells (Polacheck *et al.*, 1995); and recently to investigate the fibronectin adhesins on the yeast and hyphal-form cells (Santoni *et al.* 1995). The binding of neoglycoproteins had not been studied before the present study.

Units of all FCM measurements are arbitrary. Three FCM parameters were used namely, forward scatter (FSC) and side scatter (SSC) and fluorescence intensity. FCM requires that aggregated cells must be removed. The binding of a neoglycoprotein may also cause aggregation of *C. albicans.* and thereby influence the results, since fluorescence intensity is related to the size of the cells, or clumps of cells.

The parameters FSC and SSC allowed discrimination between two different morphologies of *C. albicans.* Germ tube formation in large number of yeast cells were recently been analyzed by Lynch *et al.* (1993), the analysis being done by germ tube-specific binding fluorescence and relative DNA content. They observed that forward and side light scatter distinguished the yeast cells from the hyphal-form cells on contour plots. DNA analysis by FCM showed DNA content of two forms of *C. albicans* which were also related to cell size (Lynch *et al.*, 1993). These authors also compared the fibrinogen-binding protein on the hyphal-form cell, on the assumption that germ tube production by the yeast cell increased the amount of fibrinogen-binding proteins. They were also able to use a cell sorter to separate the cells according to size. The contour plots in my experiments showed a similar pattern to the foregoing in that cell size and granularity increased during germ tube production.

Binding of the fucose probe to the cells was therefore confirmed by flow cytometry and, during germ tube production, the amount of bound probe increased. The lactose probe, which was used as a negative control did not bind to the cells, as shown by fluorescence intensities similar to the unlabelled cells.

To compare the virulence of yeast cell forms and hyphae of *C. albicans*, some investigators have used a single strain grown under different conditions to produce morphological variants. In the present work, the yeast cells in 199 Medium at 37°C, pH 4.0, and at 22°C, pII 6.7 did not produce germ tubes. Contour plots of the cells in the flow cytometry gave a constant pattern during the 2 h incubation that leads to germ tube production under these conditions. Fluorescence intensity of the the fucose labelled-yeast forms was not increased over the hyphal-form of cells from 199 Medium at 37°C, pH 6.7, or in Glucose-Glycine Medium at 37°C, pH 7.2. In conclusion, it was elongation of the germ tubes which gave the increases in fucose probe-binding.

Other probes, notably mannose, glucose, galactose and lactose were also tested for labelling of the hyphal-form cells and gave little binding, thereby confirming the image analysis and spectrofluorimetry results. The ions, Ca^{++} and Mg^{++} at physiological calcium concentration in blood did not show any effect on the binding of fucose probe in flow cytometry, thereby confirming the results of spectrofluorimetry. The lack of effect of calcium was supported by the lack of effect of EDTA. With buccal epithelial cells, Critchley (1986) showed that Ca^{++} and Mg^{++} enhanced slightly the adherence of *C. albicans* 2346. There was also a slight effect of cations on *C. albicans* 2346 adherence to acrylic surfaces (McCourtie and Douglas, 1981). It was also reported that Ca^{++} ions were required to maintain stable binding of iC3b to *C. albicans* pseudohyphae (Spölt *et al.*, 1993). Recently, Klotz *et al.* (1993) reported that the effect of Ca^{++} ions is on a binding structure on *C. albicans*. They observed that adherence of *C. albicans* to the extracellular matrix proteins type I collagen and fibronectin was particularly dependent on calcium.

Four different buffers at pH 5.0 and pH 7.2 showed no differences when the quantity of cell-labelled by the fucose probe was determined by flow cytometry. This

was in contrast to previous experiments by spectrofluorimetry. However, this could be due to more quenching of the fucose probe at the lower pH. Although the probe at pH 7.2 was bound to the cells less, quenching of the probe was not a problem at this pH.

Morphological mutants may also be used to detect fucose-specific lectin-like adhesins. For comparison of light scattering and fucose binding on the yeast cells during germ tube production, a germ-tube deficient mutant and its wild type strain were analyzed by contour plots and histograms (Lynch *et al.* 1993).

In the present work a spontaneous yeast mutant strain MM2002, selected by Cannon (1986), and its wild-type strain MEN were used. The mutant and its wild type were analysed by flow cytometry to identify lectin-like adhesins on the cell surfaces. Although Cannon found this strain to be strictly a yeast, other workers observed that MM2002 produced a low percent of hyphae *in vitro* (Ryley and Ryley, 1990; Cutler, 1991). In the studies of Ryley and Ryley (1990), mutant strains under germ-tube production conditions of the yeast cells contained less lectin-like adhesins during 2 h incubation time compared to the wild type. The MM2002 strain was just as lethal for mice as its germinated a wild-type strain MEN. However disease produced by the mutant was slower and correlated with initial infection which was limited to the renal cortex, as compared to wild-type strains that infected the cortical and medullary areas of the kidney during early disease. In my experiments the mutant strain from Cannon (1986) might therefore be producing a low percentage of germ tubes.

In inhibition studies, a high concentration L-fucose was non-inhibitory at concentrations up to 25 mg ml^{-1}. On the other hand, the fucose-BSA not labelled with FITC (at 600 $\mu g\ ml^{-1}$) inhibited binding of the fucose probe. Inhibition at 50% was observed with a 10-fold molecular excess of unlabelled fucose probe. The same amounts of unlabelled galactose probe showed no inhibition of binding of the fucose. This indicates that *C. albicans* 2346 lectin-like adhesins molecules are specific for fucose-BSA. Previous studies in our laboratory showed that yeast-form *C. albicans* GDH 2346 contains L-fucose-specific lectin-like molecules (Critchley and Douglas 1987b). There may therefore be two mechanisms and the combining site of the lectin-

like proteins on the *C. albicans* may be larger than a simple monosaccharide. Many mammalian lectins bind complex oligosaccharide structures, and so it is not suprising that their binding sites may accommodate several types of monosaccharide or fucose-containing oligosaccarides.

The binding of BSA-FITC without the fucose could be explained by there being a sufficient number of fluorescence residues on this probe with specificity for albumin adhesins on hyphal form *C. albicans*, as reported by Page and Odds (1988). Although the neoglycoproteins with galactose and lactose also contain BSA, they did not attach to the cells. They were therefore better negative controls than BSA-FITC since perhaps their molecular structure is different from BSA-FITC. Overall, my results confirm that fluoreceinylated neoglycoproteins prepared according to Monsigny *et al.* (1984) have specificities which are affected by amount of sugar residues.

By quantitative analysis it was found that the association of neoglycoproteins to cells could be related to their fluorescein and sugar contents. The amount of cell-associated neoglycoproteins was significantly higher with neoglycoproteins substituted with sugars than with unsubstituted neoglycoproteins. The binding of Fuc-BSA-FITC was consistently higher at 37°C than at 4°C, suggesting that only metabolically active organisms adhere as previously been found for adherence of bacteria .

Although the flow cytometry procedure is simple and reliable, aggregation of hyphal-form *C. albicans* may increase fluorescence intensity of the cell labelled with fucose containing neoglycoproteins. Similarly, virulence analysis of yeast and hyphal forms is difficult because of inadequate methods for preparating comparable inocula. The phenomenon of aggregation, by increasing the mass of the yeast cells, may enhanced their adherence to tissue as well as protecting them in the body from attack by host defences. Aggregated yeast cells can be induced *in vitro* to form germ tubes in 199 Medium at 37°C. Such tubes often are sticky, resulting in clumps of hyphae in non uniform suspension. To minimize this, several substances were used by Rahary *et al.* (1985) also were also tested in my experiments: glutathione (25mM), L-cysteine (30mM), dithiothreitol (0.5mM) ascorbic acid (30mM). These were added in 199

Medium to allow production of germ tubes by yeast cells at 37°C for 2 h. The cells were washed twice in 0.1 M citrate buffer pH 5.0. Other experiments were done with higher concentrations: glutathione (60mM), L-cysteine (60mM), dithiothreitol (60mM) and ascorbic acid (30mM). The suspension were counted for aggregated cells over 10 fields. The latter experiment was more effective than the former one, particularly with the cells in the buffers containing glutathione (60mM), dithiothreitol (60mM) or ascorbic acid (30mM). But the cells in the citrate buffer at pH 5.0 containing the substrates were labelled by the fucose probe, whereas the cells in the buffers containing glutathione (60mM) or dithiohreitol (60mM) did not bind the probe. The cells with ascorbic acid (30mM) and L-cysteine (60mM) did not prevent binding of the fucose probe. The labelling procedure increased aggregation of the hyphal-form cells.

5.3.2.4. General applications of neoglycoproteins

One of the themes in these studies has been to compare spectrofluorimetry, image analysis and flow cytometry for the binding of neoglycoproteins to *C. albicans*. These three techniques each have distinct characteristics and are complementary. Flow cytometry has the advantage over spectrofluriometry in its rapidity and convenience, and is not needing a protease as part of the procedure.

Some of the systems in which these probes have been used are summarized in Table 12. The label was most commonly, fluorescein but ^{125}I, collodial gold or latex minibead have also been used.

Table 12. Some examples of the use of neoglycoproteins to investigate adhesins on cell surfaces

Cell Type	Neoglycoproteins		Method	Author
	Sugar	Label		
Agrobacterium tumefaciens	α–Fucopyranosyl α–D-galactopyranosyl α–D-glucopyranosyl β–L-rhamnopyranosyl α–D-mannopyranosyl β–*N*-acetlglucosaminly GalNAc β–di-*N*-acetyl-chitobiosyl	Fluorescein	Spectrofluorimetry	Depierreux *et al.* (1991)
Saccharomyces cerevisiae	α–D-galactopyranosyl α–D-fucosyl α–D-mannopyranosyl	Fluorescein	Microscopy Cytofluorimeter Spectrofluorimeter	Masy *et al.* (1992)
Rhizobium lupini	di-*N*-acetyl-β-chitibiosyl α-L-fucopyranosyl α-galactopyranosyl N-acetyl-β-galactopyranosaminyl α-galactopyranosyl-6-phosphate β-glucopyranosyl	Fluorescein	Spectrofluorimeter	Wisniewski *et al.* (1994)
Mouse leukemia L1210	α–L-fucosylate α–L-rhamnopyranoside α–D-mannopyranoside	Fluorescein	Flow cytometer	Monsingy *et al.* (1984)

continued....

Toxoplasma gondii tachyzoites	galactosamide glucosamide lactosyl BSA	^{125}I	γ spectormeter Densitometry Scanning	Robert *et al.* (1991b)
Conidiobolus obscurus	α–mannosyl α–glycosyl α–L-rhammosyl α–*N*-acetylgalactosaminyl β–*N*-acetylglucosaminyl β-*N*-acetylglucosaminyl	Fluorescein Collodial gold	Microscopy	Latgé *et al.* (1988)
Plasmodium berghei *Plasmodium chabaudi*	β–*N*-acetylglucosaminyl α–Glucose-BSA β–Galactose-β4Glucose- α–D-glucose α–D-galactose α–L- fucose α–D–mannose α–L- rhamnose	Fluorescein	Flow cytometry Microscopy	Schrevel *et al.* (1986)
Bovine spermatozoa	β–D-lactose β–D-mannopyranoside α–L-fucopyranoside β–D-xylopyranoside *N*-acetyl-β–D-glucosamine *N*-acetyl-β–D-galactosaminide β–D-glucuronide	Fluorescein	Microscopy	Sinowatz *et al.* (1988)

5.4. FIBRONECTIN ADHESINS ON HYPHAL-FORM *C. ALBICANS*

Fibronectin is a large glycoprotein that is involved in many interaction with mammalian cells and microorganisms. The word means fibro (=fibrin) and nectin (Latin for: to bind). It is a dimer, with each subunit of 220 kDa made up of repeating homologous sequences. The high molecular weight was noted in the present studies when the purity of the protein was checked by SDS-PAGE prior to gold-labelling. The molecule consists of 2,300 aminoacids per unit and has 5% carbohydrate. Fibronectin is found in plasma, cerebrospinal fluid, amniotic fluid, seminal fluid, saliva and inflammatory exudation. Many cell types synthesise and secrete fibronectin, including the mucosal surfaces of mouth and vagina. The normal concentration of fibronectin in plasma is in the range of 250-600 $\mu g\ ml^{-1}$.

Fibronectin binds to a variety of bacteria, including *Staphylococccci* and *Streptococci* (Kuusela, *et al.*, 1984), *Escherichia coli* (Olsen *et al.*, 1989) and *Treponema palladium* (Thomas, *et.al .*, 1986) and also to *Saccharomyces cerevisiae* (Van De Water *et al.*, 1983) and *C. albicans* (Skerl *et al.*, 1984; Scheld *et al.*, 1985; Kalo *et al.*, 1988).

The initial idea that cellular interactions of fibronectin with bacteria are beneficial, promoting phagocytosis and clearance by is adherent properties, has largely been supplanted by the idea that binding of microorganisms to fibronectin is a step in their pathogenesis and the very opposite of beneficial to the host.

In the present experiment, binding of fibronectin to yeast and hyphal form of *C. albicans* was monitored by transmission electron microscopy using 20 nm gold particles bound to fibronectin (Au_{20}-Fn), as used by Pesciotta Peter and Moster (1987). The preparation of the colloidal gold and the labelling of fibronectin are quite simple procedures and the use of gold particles as an electron dense marker allows for easy identification of the fibronectin-labelled cells. The reliability of the technique and its

wide range of applications in transmission electron microscopy have been clearly demonstrated (Geoghegan and Ackerman, 1977; Horisberger and Rosset, 1977).

In my work, Au_{20}-Fn when added to yeast cells grown on Yeast Nitrogen Base Medium containing Galactose (YNBGal) and Modified Lee Medium without amino acids (MLMwAA) did not show any fibronectin coated gold particles on the their surface. The yeast cells grown in 199 Medium (pH 6.7) at 37°C for 1h and 2h under conditions for production of germ tubes had Au_{20}-Fn particles on the germ tube portion of hyphal-form cells. Control experiments were done with PEG-coated gold particles which showed little affiniy for the germ tube portion of hyphal-form cells. One of the most significant observation in my work was the highly specific labelling of the germ tube portion of hyphal form *C. albicans* by the Au_{20}-Fn particles. In contrast, there was few Au_{20}-Fn particles on the yeast-cell portion of the hyphal form *C. albicans*. This extends the work of Douglas and Lancaster (personal communication) who observed binding of ^{125}I Fibronectin to *C. albicans* GDH 2346 yeast cells at pH 7.0.

Klotz *et al.* (1993) have recently observed that calcium is required for the process of adherence of *C. albicans* yeast cells to various extracellular matrix proteins. They also obtained two proteins, 60 and 105 Kd, from yeast extracts by passing them over fibronectin or gelatin columm and eluting with EDTA. The same proteins were recovered from germ-tube forms of *C. albicans*. This observation showed that calcium was an important divalent cation for binding of fibronectin to *C. albicans*. *C. albicans* strains adhered in higher numbers to heparinized polyethylene surfaces preadsorbed with fibronectin than to non- modified heparinized polyethylene surfaces, supporting a role of fibronectin in mediating adhesion. (Rózalska *et al.*, 1995)

Different domain of fibronectin molecules may be bound to the yeast cells. The fibronectin and other extracellular matrix proteins are degraded by *C. albicans* most likely by the secreted aspartyl proteinase (Ray and Payne,1986). The 120 kDa fragment of fibronectin containing the cell binding domain and the aminoacid sequence, RGD, bound avidly to the surface of yeast cells (Penn and Klotz, 1994). But other investigators observed that the gelatin/collagen-binding domain of fibronectin is a

potent inhibitor of fibronectin binding to *C. albicans,* and also the fibrin I-, and the heparin II-binding domains also inhibit fibronectin binding, but are less active (Negre *et al.*, 1994). Their results also indicated that interactions with the cell-binding domain are not mediated by the Arg-Gly-Asp or other known recognition sequences. Santoni *et al.* (1995) showed that *C. albicans* expresses a fibronectin receptor antigenically related to α5β1 integrin. A monoclonal antibody (MAb) directed against human α5β1 subunit positively bound to the yeast and hyphal-forms *C. albicans* by immunofluorescence and flow cytometric analysis. This immunoreactivity increased upon germ tube production. Both forms bound to the Arg-Gly-Asp (RGD) containing 120-kDa fragment of fibronectin and adhesion to intact fibronectin was markedly inhibited by Gly-Arg-Gly- containing peptide.

Interest in the study of adherence of *C.albicans* to various surfaces has increased in recent decades. Adherence of microorganisms to various surfaces throws light on mechanism of pathogenesis and suggest means of controlling infection before their onset. In the opportunistic dimorphic fungal pathogen, *C. albicans,* the wall not only maintains the structural shape which characterizes each growth form, but is also the site of the initial interaction between the organism and its environment.

5.5. ADHESINS AND VIRULENCE

Adherence of *C. albicans* to host cells or tissue occurs, as in many infectious diseases. With *C. albicans*, the outermost layer of the cell wall, containing the mannoproteins, is believed to be the most likely candidate for putative fungal adhesins. The relationship between the fungus and human cells has been explored by the reduction of adherence by carbohyrate, in one experiment, and by a peptide or protein in the other. This indicates that multiple mechanisms of adherence to tissue and epithelial cells may take place with the same organism (Pendrak and Klotz, 1995).

Critchley and Douglas (1987a, b) demonstrated a lectin-like receptor with specificity for *N*-acetyl-D-glucosamine. Brassat *et al.* (1991) used the human milk oligosaccharides probe Fuc α1-> 2Galβ determinant (the H sugar sequence found on all blood group substances of the ABO[H] system) to inhibit the adhesin of *C. albicans* to buccal epithelial cells, suggesting that disaccharide-bearing cell surface glycoconjugates could act as host receptors. The effects of various lectins on hepatic trapping and killing of *C. albicans* by the isolated perfused mouse-liver model was explored by Sawyer *et al.* (1992b). Their data suggested that a fucose-containing receptor on the surface of either sinusoidal endothelial cells or Kupffer cells were involved in the trapping of *C. albicans* by the perfused mouse liver. Moreover, lectins with binding specificities for mannose, *N*- acetylgalactosamine, and galactose increased hepatic killing of *C. albicans.*

Evidence that the receptor on BEC is larger than L-fucose, or that a particular stereochemical configuration is necessary was produced by Tosh and Douglas (1992). Yu *et al.*, (1994a) described the purification and initial characterization of a fimbrial adhesin from *C. albicans*.. The major structural subunit was a glycoprotein of 80 to 85% carbohydrate (containing principally D-mannose) and 10 to 15% protein. The molecular weight of the glycosylated fimbrial subunit was approximately 66,000 while the unglycosylated protein had an approximate molecular weight of 8,644. In a subsequent paper, the fimbriae were reported to interact with glycosphingolipid receptors via a carbohydrate portion, since the fimbriae bound to synthetic β-GalNAc(1-4)β-Gal-protein conjugates and the disaccharide inhibited binding of fimbriae to BECs (Yu *et al.*, 1994b). The authors concluded that the *C. albicans* yeast form expresses a fimbrial adhesin that binds to glycosphingolipids displayed on the surface of human BECs. Recently, Cameron and Douglas (1996) confirmed these findings in a system with biotinylated yeasts to lipids combining with glycosphingolipids extracted from human BEC and separated on thin-layer chromatograms. Binding was visualized by the addition of ^{125}I-streptavidin followed by autoradiography. The strain used, *C. albicans* GDH 2346 (as in my work), had a

specificity for the fucose-containing receptors in the lipid extracts. A parallel chromotogram overlaid with biotinylated *Ulex europaeus* lectin, which is a fucose-binding lectin with a specificity for H blood group antigen, showed that two of the glycosphingolipids carried this antigenic determinant. The lipid extract from BEC containing *N*-acetylgalactosamine also acted as a receptor for five strains of *C. albicans*. This component was clearly different from the fucose-binding adhesin, which showed no affinity for this glycolipid.

As will be seen from the above, most adhesion experiments with *C. albicans* have been done with the yeast-cell form of the fungus and not with the hyphal forms as in this thesis. The dimorphic transition of *C. albicans* from yeast cells to hyphal form is a complex event and both morphologies are found in tissues in candidosis. Thus information on the identity, composition, and distribution of the cell wall adhesins of both forms is important. This studies in this thesis, by using hyphal-form *C. albicans*, allowed identification and quantitation of the lectin-like adhesins on the germ tube portions.

The interaction of mannoprotein of yeast cells with fucose-containing moieties on epithelial cells may be important for the correlation of candidal carriage with blood group O (Burford-Mason *et al*, 1988, Thom *et al.* 1989). Hosts with blood group O express (on their buccal and vaginal epithelial cells) the H antigen which terminates in α-1,2-fucose linked to D-galactose, *N*-acetyl-D-glucosamine, and *N* -acetyl-D-galactosamine .

Fucose-containing receptors are also involved with other micro-organisms. Adhesion of *Streptococcus pyogenes* to pharyngeal epithelial cells was sensitive to inhibition by free galactose and fucose (Tylewska *et al.*, 1988). Fluorescein isothiocyanate-labeled *Helicobacter pylori* strains were bound to surface mucous cells present in the pit region of human and rat gastric units, and binding was abolished by a rich source of fucosylated carbohydrates (Falk *et al.* 1993). Wang and Stinson (1994) also showed that binding of M6 protein of *S. pyogenes* to human epithelial cells was highly selective for certain fucose-containing oligosaccharides.

Weinmeister and Dal Nogare (1994) compared respiratory epithelial cell-surface carbohydrate levels of normal subjects with those of critically ill patients. Lectins were used to quantitate the amount of mannose, galactose, fucose, and sialic acid on buccal and tracheal cells. Buccal cells of severely ill patients had decreased amounts of sialic acid and galactose, but unchanged levels of fucose compared with those of normal subjects. The loss of sialic acid and galactose may therefore explain the high prevalence of gram-negative bacterial colonization and pneumonia in the critically ill.

5.6. POSSIBLE FUTURE STUDIES

With fucose-containg structures being present on epithelial cells, it is possible that neoglycoproteins such as Fuc-BSA could be the basis of a therapeutic treatment of candidosis. As an extension of this, the neoglycoproteins with drug substituents might serve as carriers of antifungal agents, as previously described by Monsigny *et al.* (1994).

Since fucose alone may not have sufficient specificity, studies similar to those described here should be done with fucose-containing di- and oligo-saccharides, both alone and coupled to protein and lipid. Both of the latter could also be varied, e.g. by coupling fucose to proteins other than BSA.

Additional *C. albicans* mutants which lack adhesion to buccal epithelial cells could be used for further characterization of lectin-like adhesins (Fukayama and Calderone, 1991). In my work, the binding of neoglycoproteins to *C. albicans* was investigated only *in vitro* , but it could also be studied *in vivo* with animal models. The *in vivo* binding of neoglycoproteins could be explored by methods like those of Robert *et al.* (1991a) with yeast and hyphal forms of *C. albicans* . They showed binding of fibrinogen to various *C. albicans* strains by immunofluorescence on kidney section of experimentally infected mice.

The purification of fucose-binding protein from yeast cell and germ tube portion of hyphal *C. albicans* would be a further topic to parallel the studies of the streptococcal M6 protein by Wang and Stinson (1994). Isolation of the genes encoding this protein on the two forms of yeast cell might provide new insights into the role of lectin-like proteins in candidal adhesion.

Definition of the mechanisms of attachment of *C. albicans* may have important therapeutic implications. New therapeutic strategies for the treatment of candidosis may involve inhibition of attachment of the organism to the host cell. The apparent correlation of fibronectin with clinical invasiveness supports the idea that adherence of *C. albicans* to fibronectin (present in blood clots or subendothelial matrix, or coating artificial surfaces such as catheters, cardiac shunts, and valves), could play an important role in the pathogenicity of the organism (Penn and Klotz, 1994; Rozalska, *et al.* 1995). Finally, numerous human proteins such as fibrinogen, fibronectin and laminin that contain the RGD sequence may be involved in the interactions of *C. albicans* with inert surfaces such as plastic. The study of such interactions would have implications in the practical management of patients with prostheses made with these materials.

6. APPENDICES

Appendix 1. Cultural and biochemical characteristics of *Candida* species frequently isolated from clinical specimens (based on data from Warren and Shadomy, 1991)

Species	Germ tube production	Chlamydospores	Assimilation												Fermentation					
			Glucose	Maltose	Sucrose	Lactose	Galactose	Melibiose	Cellobiose	Inositol	Xylose	Raffinose	Trehalose	Dulcitol	Glucose	Maltose	Sucrose	Lactose	Galactose	Trehalose
Candida albicans	+	+	+	+	+	-	+	-	-	-	+	-	+	-	F	F	-	-	F	F
C. guilliermondii	-	-	-	+	+	+	-	+	+	+	-	+	+	+	F	-	F	-	F*	F*
C. krusei	-	-	+	-	-	-	-	-	-	-	-	-	-	-	F	-	-	-	-	-
C. lipolytica	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. lusitaniae	-	-	+	+	+	-	+	-	+	-	+	-	+	-	F	-	F	-	F	F
C. parapsilosis	-	-	+	+	+	-	+	-	-	-	+	-	+	-	F	-	-	-	-	-
C. pseudotropicalis	-	-	+	-	+	+	+	-	+*	-	+*	+	-*	-	F	-	F	F*	F	
C. rugosa	-	-	+	-	-	-	+	-	+*	-	+	-	-	-	-	-	-	-	-	-
C. stellatoidea	-	-	+	+	-	-	+	-	+	-	+	-	+	-	F	F	F	-	F	
C. tropicalis	-	-	+	+	+	-	+	-	+	-	+	-	+	-	F	F	F	-	F	F

* Strain variations;

F the sugar is fermented (i.e. gas is produced).

\+ growth greater than negative control

\- neither growth over control nor fermentation

Appendix 2. Composition of 199 Medium

(from Flow Laboratories, Irvine, Scotland)

Ingredient	mg l^{-1}
L-Alanine	25.00
L-Arginine+HCl	70.00
L-Aspartic acid	30.00
L-Cysteine	0.09871
L-Cystine disodium salt $2H_2O$	25.16
L-Glutamic acid	66.82
Glutathione	50.00
Glycine	21.88
L-Histidine+HCl+$2H_2O$	10.00
L-Hydroxyproline	20.00
L-Isoleucine	60.00
L-Lysine+HCl	70.00
L-Methionine	15.00
L-Phenylalanine	25.00
L-Proline	40.00
L-Serine	25.00
L-Threonine	30.00
L-Tryptophan	10.00
L-Tyrosine disodium salt.$2H_2O$	57.66
L-Valine	25.00
Ascorbic acid	0.05
Biotin	0.01
Calciferol	0.10
D.Ca.Pantothenate	0.01
Choline chloride	0.50
Folic acid	0.010
I-Inositol	0.05
Menaphthone sodium bisulfite.$3H_2O$	0.019
Nicotinic acid	0.025
Nicotinamide	0.075
p-Aminobenzoic acid	0.05
Pyridoxal.HCl	0.025

Pyridoxine.HCl	0.025
Riboflavin	0.01
Thiamin.HCl	0.01
DL-α Tocopherol phosphate disodium salt.$2H_2O$	0.0106
Vitamin A acetate	0.14
$CaCl_2.2H_2O$	185.5
$Fe(NO_3)_3.9H_2O$	0.72
KCl	400.00
KH_2PO_4	60.00
$MgSO_4.7H_2O$	200.00
NaCl	8000.00
HEPES (20mM)	
Na_2HPO_4	47.50
Adenine sulphate.$2H_2O$	10.98
5-AMP.H_2O	0.2104
ATP.disodium salt.$3H_2O$	1.098
Cholesterol	0.20
2-Deoxyribose	0.50
D-Glucose	1000.00
Guanine.HCl	0.30
Hypoxanthine	0.30
D-Ribose	0.50
Sodium acetate	50.00
Phenol red sodium salt	7.00
Thymine	0.30
Tween 80	20.00
Uracil	0.30
Xanthine	0.30

Appendix 3. Buffers

Appendix 3A. 0.01M Borate buffer (pH 8.1)

Borate (0.9525g) was dissolved in 100 ml of DW. 0.1 M HCl (19.7 ml) was added to 50 ml of borate solution to prepare 0.025 M Borate-HCl buffer and finally was diluted with 150 ml of DW.

Appendix 3B.Tris buffers

a. 0.05 M Tris hydrochloride containing 0.8 M Mannitol (pH 7.5)

Tris (3.0278 g) and mannitol (72.84 g) were dissolved in 300 ml DW and the pH was adjusted with HCl to pH 7.5.

b. 0.1 M Tris hydrochloride (pH 7.2)

Tris (1.211 g) was dissolved in 100 ml DW. Tris solution (50 ml) was mixed with 0.1 M HCl (44.7 ml) and 6.3 ml DW.

c. 0.1 M Tris hydrochloride and 15 mM NaCl (pH 7.4)

Tris (1.211 g) was dissolved in 100 ml DW. Tris solution (50 ml) was mixed with 0.1 M HCl (42.0 ml) and added NaCl (0.0876 g) and DW to 100 ml.

Appendix 3C. 0.1M Citrate buffer (pH 5.0)

$C_6H_8O_7.H_2O$ (2.101 g) and $C_6H_5O_7Na_3.H_7O$ (2.941 g) were separately dissolved in 100 ml DW. The former solution (35 ml) was added to $C_6H_5O_7Na_3.H_7O$ solution (65 ml).

Appendix 3D. 0.1 M Phosphate buffer (pH 7.2)

KH_2PO_4 (1.36 g) and NaOH (0.4 g) were separately dissolved in 100 ml DW, 50 ml of the former was added to 34.7 ml of the latter NaOH solution followed by 12.6 ml DW.

Appendix 3E. 0.1 M Sodium acetate buffer (pH 5.0)

$C_2H_3O_2Na.3H_2O$ (0.8204 g) and acetic acid (0.6005 g) were dissolved in separate 100 ml portions of DW and 67.8 ml of the former solution was added to 32.2 of the latter.

Appendix 3F. 0.15 M Phosphate-saline buffer (pH 7.4)

One tablet Dulbecco 'A' (Oxoid) was dissolved in 100 ml DW.

All buffers were filtered by 0.45µ filter before used.

Appendix 4. Spectrofluorimetry analysis of *C. albicans*

Appendix 4.1. Calculation of the fucose probe binding sites on hyphal-form *C. albicans*

Binding of the fucose probe concentration on the cells after pronase treatment (section 4.3.2.8)			23.6 µg
BSA molecular weight:	1.0 x 69, 000 MW	=	69,000.0
Fucose molecular weight:	16.0 x 164.2 MW	=	2.627.2
FITC molecular weight	2.4 x 332.0 MW	=	796.8
Total molecular weight		=	72,424.0
Avogadro's No			$6.2x10^{23}$

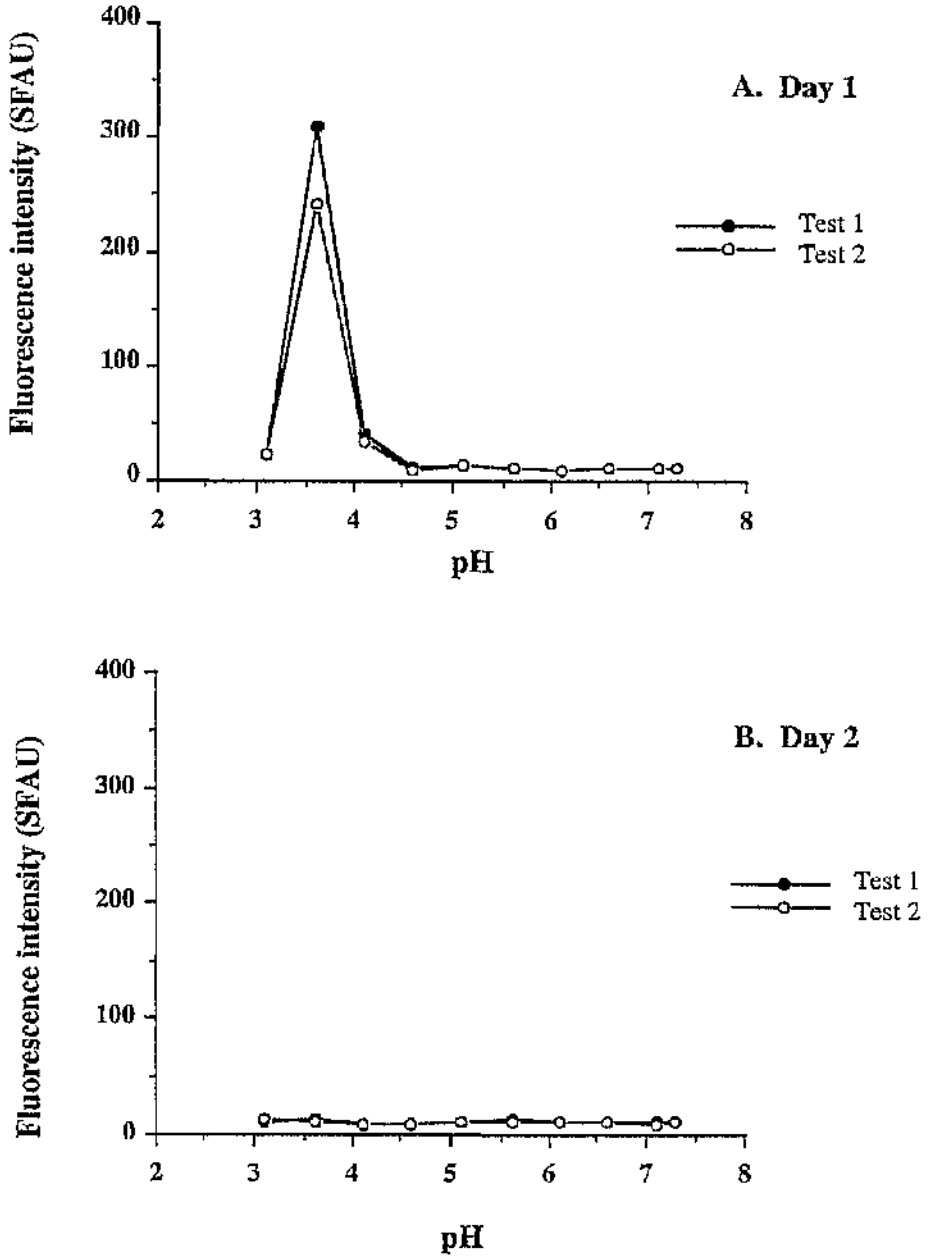
72.424.0 g probe would have $6.2x10^{23}$ binding sites.

1 µg probe would have $6.2x10^{23}$: $7.242 \times 10^4 \times 10^6$ binding sites.

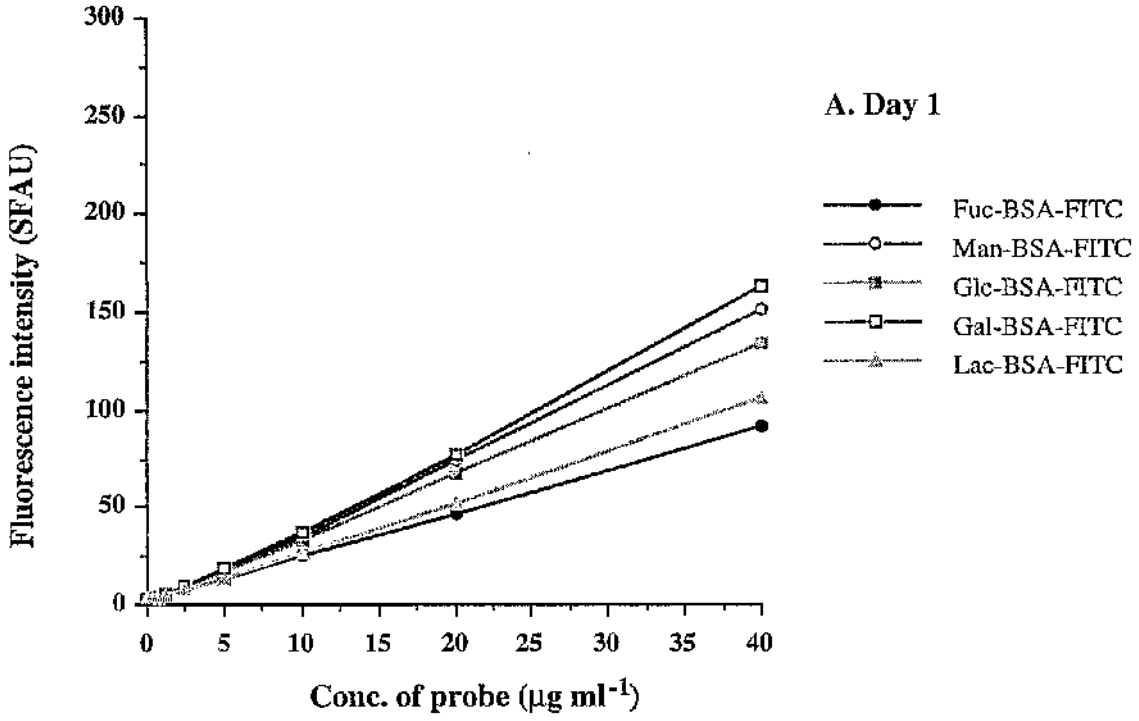
23.6 µg probe would have $23.6 \times 6.2x10^{23}$: $7.242 \times 10^4 \times 10^6$ binding sites.

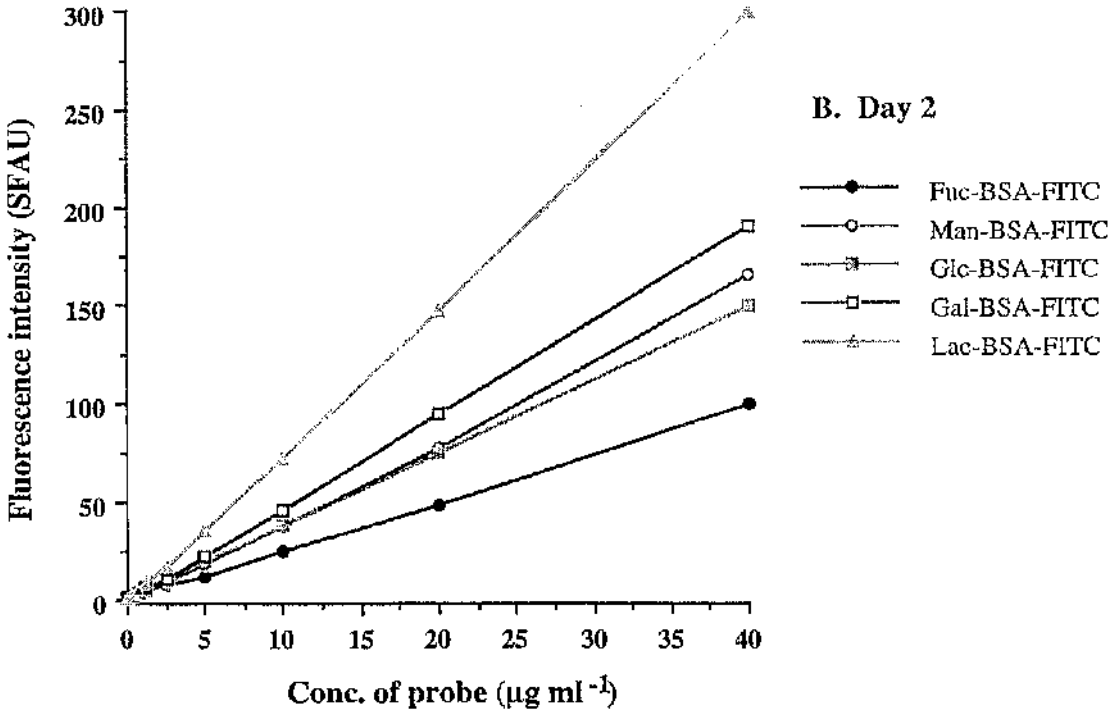
Each hyphal *C. albicans* would have $23.6 \times 6.2 \times 10^{23} : 7.242 \times 10^{10} \times 10^7$ = approximately 2.1×10^7 binding sites.

Appendix 4.2. Effect of pH on binding of the lactose probe in duplicated test on two separate days (A and B)



Appendix 4.3. Standard curves of five probes for two separate days (A and B)

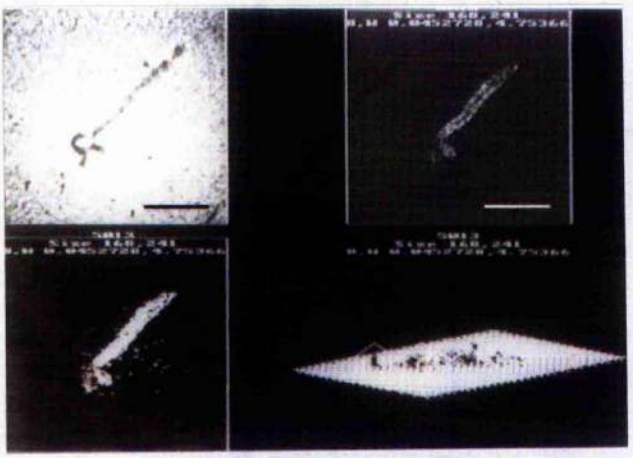




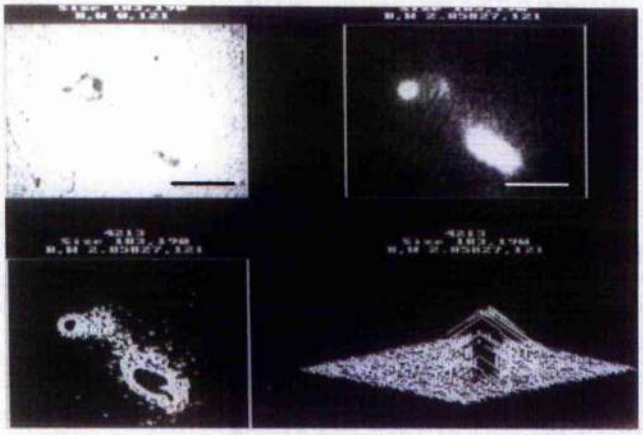
Appendix 5. Image analysis of *C. albicans*

Appendix 5.1. Printing paper computer output images of hyphal-form *C. albicans* labelled with the fucose probe 1000x (see Text 4.2.2 section for explanation). Each composite picture is of a single hyphal-form cell. Bars represent 5 μm

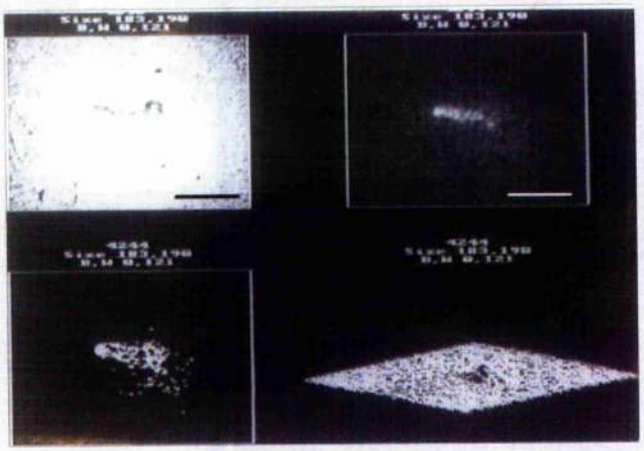
a.



b.

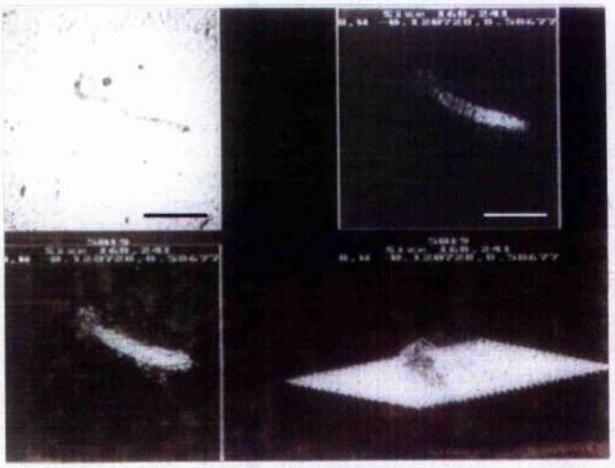


c.

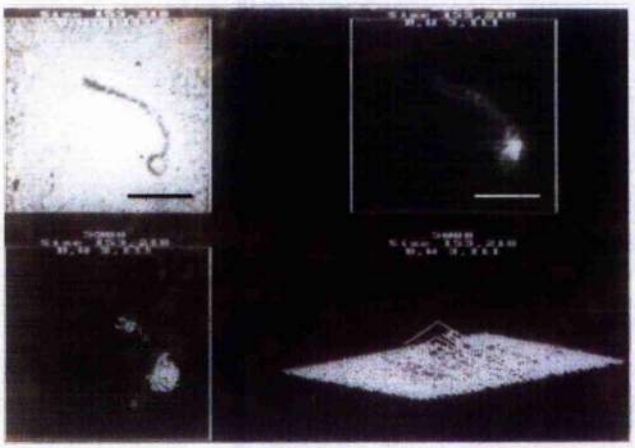


continued....

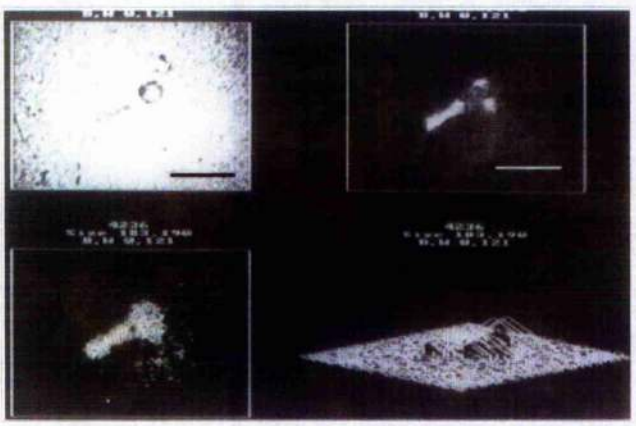
d.



e.



f.



Appendix 5.2. Image analysis data: Measurements of fluorescence intensity of six areas on yeast cell and germ tube portions of hyphal-forms of *C. albicans*

Day	Cell No	Fluorescence intensity (IAAU)					
		Germ Tube			Yeast Cell		
		1	2	3	4	5	6
1	1	17.3	17.8	35.7	70.5	9.3	15.6
1	2	9.0	11.7	8.7	3.8	3.1	4.5
1	3	7.3	3.6	4.6	1.6	1.5	1.8
1	4	10.0	6.2	0.7	20.4	8.0	6.4
1	5	14.8	12.6	11.7	2.2	2.9	7.9
1	6	18.9	8.8	11.8	14.8	5.6	4.4
1	7	12.2	7.0	0.9	0.1	0.1	0.8
1	8	5.0	6.6	9.1	13.3	1.2	2.7
1	9	13.0	15.9	13.2	8.8	4.1	3.9
1	10	2.5	4.7	4.8	3.3	2.6	1.3
1	11	4.4	19.8	2.9	1.7	1.3	3.2
1	12	25.5	4.8	3.3	6.9	4.5	4.2
1	13	6.9	20.9	8.0	2.8	2.2	4.7
1	14	7.6	17.0	7.1	0.9	0.3	0.4
1	15	0.7	6.6	9.3	4.3	5.2	6.3
1	16	4.3	6.0	8.6	7.7	6.3	8.2
1	17	2.1	6.2	36.9	1.8	0.9	0.7
1	18	0.6	2.2	5.2	3.9	2.8	2.3
1	19	3.3	4.4	3.4	4.4	1.6	2.4
1	20	10.6	10.2	14.1	2.0	1.0	1.9
1	21	3.6	4.5	3.6	0.7	2.7	2.8
1	22	0.3	4.6	6.4	2.2	2.5	3.8
1	23	4.1	7.9	35.3	4.1	5.3	12.4
1	24	15.2	8.1	5.5	8.9	8.9	9.0
1	25	8.7	9.8	2.4	2.4	1.1	1.5
1	26	9.0	13.7	5.9	1.4	2.0	2.4
1	27	2.8	9.2	5.3	2.3	1.0	1.4
1	28	5.0	9.4	8.8	4.9	6.0	5.4
1	29	0.5	4.7	3.7	2.7	2.3	2.7
1	30	1.2	9.8	10.7	3.9	3.3	7.1

Continued....

Day	Cell No	Fluorescence intensity (IAAU)					
		Germ Tube			Yeast Cell		
		1	2	3	4	5	6
2	1	17.9	22.2	1.3	6.8	2.1	2.4
2	2	30.1	18.5	-1.9	-2.7	-4.0	-3.0
2	3	24.5	26.5	23.5	5.7	2.7	5.0
2	4	15.5	25.6	17.5	26.0	17.6	10.9
2	5	62.0	30.9	18.1	6.0	7.9	15.7
2	6	24.4	36.7	53.6	59.2	100.0	95.0
2	7	7.0	28.7	13.7	23.3	10.3	6.0
2	8	14.7	24.3	26.6	14.5	17.7	16.6
2	9	14.0	23.2	22.7	11.0	14.1	19.7
2	10	17.8	35.2	41.7	12.3	19.9	28.2
2	11	19.5	35.0	31.2	13.4	18.6	22.3
2	12	31.5	45.0	26.9	12.6	11.0	12.7
2	13	24.9	31.3	28.6	10.6	9.4	9.8
2	14	16.2	8.4	8.8	-1.9	2.7	-0.3
2	15	22.6	28.3	32.1	11.8	9.8	9.3
2	16	50.2	34.2	27.9	6.6	3.1	0.5
2	17	5.2	16.8	23.1	8.2	10.5	9.8
2	18	13.1	25.4	26.4	26.1	22.4	21.8
2	19	75.3	36.6	21.9	40.0	6.4	6.4
2	20	33.5	29.7	23.0	6.8	5.5	4.8
2	21	26.2	23.4	18.3	3.8	2.0	7.1
2	22	31.1	41.4	10.1	4.6	0.5	10.6
2	23	14.0	22.7	26.6	7.7	8.8	9.1
2	24	1.7	6.1	10.6	10.8	13.8	12.9
2	25	-1.1	0.8	4.0	-5.1	-1.5	0.8
2	26	4.3	8.4	13.0	53.6	65.5	95.4
2	27	17.1	22.0	17.8	7.2	7.4	12.2
2	28	5.2	12.1	13.8	15.2	19.8	15.9
2	29	21.7	18.6	4.8	5.7	3.1	6.4
2	30	14.0	8.5	7.6	10.9	13.1	11.9

Continued....

Day	Cell No	Fluorescence intensity (IAAU)					
		Germ Tube			Yeast Cell		
		1	2	3	4	5	6
3	1	4.9	26.6	7.1	-1.0	1.2	5.3
3	2	10.0	11.0	5.8	7.6	3.9	3.6
3	3	11.7	6.9	2.2	5.2	-1.1	0.4
3	4	0.8	3.9	10.1	20.3	14.3	22.2
3	5	15.9	22.1	34.9	21.0	18.6	37.8
3	6	5.1	68.4	79.6	14.8	39.3	18.6
3	7	78.5	18.6	16.7	11.5	6.3	14.1
3	8	10.1	19.9	25.4	36.7	38.3	24.2
3	9	0.1	5.7	5.8	-0.4	1.2	4.6
3	10	15.3	25.9	19.8	7.1	6.2	6.2
3	11	1.4	8.8	16.8	10.6	7.6	7.0
3	12	2.9	7.3	11.8	4.1	4.6	5.4
3	13	3.4	4.4	10.9	9.4	11.0	9.3
3	14	0.6	10.8	7.0	7.3	6.9	8.1
3	15	4.1	14.9	27.5	8.2	2.0	3.9
3	16	0.7	7.7	11.2	10.6	8.5	5.0
3	17	30.5	27.5	24.2	5.3	8.6	7.8
3	18	55.5	47.5	37.3	35.0	14.5	37.3
3	19	11.8	20.9	17.2	20.6	9.2	8.2
3	20	4.1	1.6	6.0	5.8	11.0	10.2
3	21	10.5	13.6	2.8	2.3	2.4	2.7
3	22	34.6	23.0	17.9	5.2	5.4	12.8
3	23	11.1	23.8	14.0	14.4	4.0	6.4
3	24	8.4	4.1	1.4	3.7	0.9	1.7
3	25	20.1	27.2	16.3	1.1	1.4	1.5
3	26	9.2	18.6	16.7	7.8	9.0	10.4
3	27	10.1	9.9	7.2	8.2	8.1	8.1
3	28	20.9	22.8	6.2	4.8	3.0	4.2
3	29	1.0	3.5	6.2	4.8	3.0	3.2
3	30	7.9	7.1	25.8	15.6	16.7	13.9

Appendix 6. Flow cytometry analysis of *C. albicans* : Data from histograms showing percentage of the cells labelled with various probes, and their fluorescence intensity (FCAU) with marker limits as Log_{10} from 1.76 to 4.0

Appendix 6.1. Analytical data from Figure 38

Time of	Percentage of the cells and their mean fluorescence intensity (FCAU)					
incubation	Fuc[a]		Lac[b]		BG[c]	
(h)	%[d]	FI[e]	%	FI	%	FI
	21.2	79.5	11.6	40.1	9.4	33.9
0	29.4	188.0	14.2	43.0	7.8	30.6
	63.1	363.6	9.4	52.3	8.8	51.5
	47.7	252.4	6.2	31.2	8.7	47.1
Mean	**40.3**	**220.8**	**10.3**	**41.6**	**8.6**	**40.7**
±SEM	**±9.2**	**±59.4**	**±1.6**	**±4.3**	**±1.6**	**±5.0**
	33.3	104.5	13.9	36.5	14.8	42.4
1	31.3	248.5	5.7	47.2	15.8	46.2
	76.6	495.1	14.0	53.2	12.1	44.8
	71.9	370.8	17.4	40.9	7.7	35.4
Mean	**53.3**	**304.7**	**15.2**	**44.4**	**12.6**	**42.2**
±SEM	**±12.1**	**±87.5**	**±0.8**	**±4.2**	**±1.8**	**±2.3**
	35.7	125.5	21.3	40.5	14.2	40.4
2	42.8	267.2	21.4	47.7	18.8	34.9
	84.2	415.0	17.3	38.6	4.5	74.9
	85.1	405.3	13.0	32.0	5.2	58.2
Mean	**61.9**	**303.2**	**18.5**	**39.7**	**10.6**	**52.1**
±SEM	**±13.1**	**±68.1**	**±1.9**	**±3.2**	**±3.4**	**±9.0**

[a] Fuc-BSA-FITC

[b] Lac-BSA-FITC

[c] Background

[d] Percentage of the cells

[e] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.2A. Analytical data from Figure 41

199 Medium pH 6.7 at 4˚C

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)											
Time (h)	0				1				2			
	Fuc[a]		BG[b]		Fuc		BG		Fuc		BG	
	%[c]	FI[d]	%	FI	%	FI	%	FI	%	FI	%	FI
	29.9	331.7	0.2	28.0	36.9	199.6	0.07	26.6	27.7	221.6	0.3	65.7
	28.7	349.4	2.2	54.4	34.0	336.7	0.2	21.4	28.9	165.5	0.1	20.8
	52.0	360.2	0.5	22.4	43.6	298.5	0.7	25.7	25.8	186.3	0.3	28.9
	47.7	387.1	1.5	26.1	36.6	299.9	0.5	31.0	26.6	200.0	0.4	26.9
Mean	**39.5**	**357.1**	**1.1**	**32.7**	**37.7**	**283.6**	**0.3**	**26.1**	**27.0**	**193.3**	**0.2**	**35.5**
±SEM	**±6.0**	**±12.5**	**±0.4**	**±7 .3**	**± 2.0**	**± 29.3**	**± 0.1**	**±1.8**	**±0.6**	**±11.7**	**±0.05**	**± 20.3**

[a] Fuc-BSA-FITC

[b] Background

[c] Percentage of the cells

[d] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.2B. Analytical data from Figure 41

199 Medium pH 6.7 at 22°C

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)											
Time (h)	0				1				2			
	Fuc[a]		BG[b]		Fuc		BG		Fuc		BG	
	%[c]	FI[d]	%	FI	%	FI	%	FI	%	FI	%	FI
	21.8	355.6	0.1	22.5	39.7	429.9	0.07	22.3	41.1	371.7	0.0	0.0
	17.4	278.2	0.1	21.8	30.0	305.9	0.2	31.3	42.8	423.9	0.2	190.3
	59.2	489.8	0.5	25.3	55.4	533.4	0.3	24.1	44.4	251.9	1.0	24.1
	59.8	464.8	0.2	31.7	56.1	472.2	0.7	62.5	45.0	296.0	0.6	23.9
Mean	**39.7**	**397.1**	**0.2**	**25.3**	**45.3**	**435.3**	**0.3**	**35.0**	**43.3**	**335.8**	**0.4**	**59.5**
±SEM	**±11.5**	**±49.1**	**± 0.05**	**± 4.5**	**± 6.3**	**±48.0**	**± 0.1**	**±18.7**	**±0.8**	**±38.3**	**± 0.2**	**± 43.9**

[a] Fuc-BSA-FITC

[b] Background

[c] Percentage of the cells

[d] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.2C. and 6.3A. Analytical data from Figure 41 and 43

199 Medium pH 6.7 at 37°C

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)											
Time (h)	0				1				2			
	Fuc[a]		BG[b]		Fuc		BG		Fuc		BG	
	%[c]	FI[d]	%	FI	%	FI	%	FI	%	FI	%	FI
	35.0	298.7	0.2	28.6	73.8	379.0	0.1	26.6	89.7	433.6	0.3	65.7
	28.7	349.4	0.2	54.4	75.2	378.6	0.2	21.4	88.3	386.0	0.1	20.8
	58.2	521.9	0.5	22.4	61.6	485.4	0.7	25.7	75.2	341.0	0.3	28.9
	61.3	680.5	1.5	26.1	68.9	750.2	0.5	31.0	73.5	307.9	0.4	26.9
Mean	**45.8**	**462.6**	**0.6**	**32.8**	**69.8**	**498.3**	**0.3**	**26.1**	**81.6**	**367.1**	**0.2**	**35.5**
±SEM	**±8.1**	**±86.9**	**± 0.3**	**± 7.2**	**±3.0**	**±87.6**	**± 0.1**	**±3.9**	**±4.2**	**±27.3**	**±0.05**	**± 10.1**

[a] Fuc-BSA-FITC

[b] Background

[c] Percentage of the cells

[d] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.3B. Analytical data from Figure 43

GG Medium pH 7.2

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)											
Time (h)	0				1				2			
	Fuc[a]		BG[b]		Fuc		BG		Fuc		BG	
	%[c]	FI[d]	%	FI	%	FI	%	FI	%	FI	%	FI
	30.0	360.2	0.0	20.1	81.5	428.4	1.1	27.3	85.9	892.8	0.4	29.6
	34.9	417.9	0.0	137.0	87.2	568.5	1.1	48.2	83.5	729.2	1.4	25.3
	48.6	561.3	0.1	24.1	55.9	323.7	1.9	28.6	85.9	363.8	0.8	28.3
	54.6	491.1	0.1	20.9	50.4	309.9	1.0	40.0	80.9	302.5	0.8	33.5
Mean	**42.0**	**457.6**	**0.05**	**50.5**	**68.7**	**407.6**	**1.2**	**36.0**	**84.0**	**572.0±**	**0.8**	**29.1**
±SEM	**± 5.6**	**± 43.7**	**± 0.02**	**± 28.8**	**±9.1**	**±59.7**	**± 0.2**	**± 4.8**	**±1.1**	**142.4**	**± 0.4**	**± 1.6**

[a] Fuc-BSA-FITC

[b] Background

[c] Percentage of the cells

[d] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.4A. Analytical data from Figure 45

Yeast-form *C. albicans*

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)											
	Fuc[a]		Man[b]		Glc[c]		Gal[d]		Lac[e]		BG[f]	
	%[g]	FI[h]	%	FI	%	FI	%	FI	%	FI	%	FI
	26.0	179.2	7.8	65.9	3.4	18.7	3.0	41.8	2.4	31.7	2.5	17.3
	43.4	61.4	11.7	43.6	13.7	50.6	5.3	77.3	3.2	18.9	4.3	13.0
	32.8	148.5	14.5	129.2	22.9	83.7	5.9	73.7	7.2	146.3	4.3	26.2
	31.6	248.3	8.6	162.0	9.8	158.0	6.2	43.7	5.3	84.3	5.2	34.0
	33.0	76.9	17.5	79.7	12.9	35.5	5.1	26.7	3.8	16.7	3.4	13.0
	30.2	122.9	6.0	109.3	2.7	17.3	2.6	32.1	2.5	18.9	2.1	76.1
Mean	**32.8**	**139.5**	**11.0**	**98.2**	**10.9**	**60.6**	**4.6**	**49.2**	**4.0**	**52.8**	**3.6**	**29.9**
±SEM	**±2.3**	**±28.1**	**±1.7**	**±17.7**	**±3.0**	**±21.8**	**±0.6**	**±8.6**	**±0.7**	**±21.4**	**±0.4**	**±9.7**

[a] Fuc-BSA-FITC
[b] Man-BSA-FITC
[c] Glc-BSA-FITC
[d] Gal-BSA-FITC
[e] Lac-BSA-FITC
[f] Background
[g] Percentage of the cells
[h] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.4B. Analytical data from Figure 45

Hyphal-form *C. albicans*

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)											
	Fuc[a]		Man[b]		Glc[c]		Gal[d]		Lac[e]		BG[f]	
	%[g]	FI[h]	%	FI	%	FI	%	FI	%	FI	%	FI
	64.8	322.7	60.0	100.4	45.4	53.6	41.9	43.8	23.2	49.4	22.8	28.4
	80.9	253.0	76.9	113.8	67.1	77.1	43.2	24.6	15.2	32.0	7.0	27.6
	94.5	215.2	81.4	68.6	63.3	39.3	27.7	27.7	9.1	58.0	12.6	44.6
	96.5	309.8	82.3	88.5	67.6	45.8	38.9	58.5	12.6	32.0	4.1	52.1
	78.1	263.8	76.1	118.7	65.5	73.1	47.2	31.3	15.2	52.5	7.7	23.1
	69.7	289.9	66.1	160.5	46.0	47.0	43.1	34.2	26.1	43.4	23.8	34.5
Mean	**80.7**	**275.4**	**73.8**	**108.4**	**59.1**	**55.9**	**40.3**	**36.6**	**16.9**	**44.5**	**13.0**	**35.0**
±SEM	**±5.2**	**±16.2**	**±3.5**	**±12.7**	**±4.2**	**±6.2**	**±2.7**	**±5.1**	**±2.6**	**±4.4**	**±3.4**	**±4.4**

[a] Fuc-BSA-FITC

[b] Man-BSA-FITC

[c] Glc-BSA-FITC

[d] Gal-BSA-FITC

[e] Lac-BSA-FITC

[f] Background

[g] Percentage of the cells

[h] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.5A. Analytical data from Figure 47

pH 5.0

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)									
	Citrate				Acetate					
	Fuc[a]		BG[b]		Fuc		Fuc+ Ca ++ Mg++		BG	
	%[c]	FI[d]	%	FI	%	FI	%	FI	%	FI
	85.4	324.5	5.3	38.6	55.6	99.8	80.0	127.2	12.3	46.9
	76.0	299.6	5.0	44.4	60.9	158.5	77.6	149.8	10.7	39.9
	88.3	150.1	2.7	32.9	84.9	113.2	83.5	110.8	4.0	37.4
	87.7	149.8	3.4	41.6	82.7	135.2	93.4	276.5	5.6	76.2
Mean	**84.3**	**231.0**	**4.1**	**39.3**	**71.0**	**126.6**	**83.6**	**166.0**	**8.1**	**50.0**
±SEM	**±2.8**	**±47.0**	**±0.6**	**±2.4**	**±7.4**	**±12.8**	**±3.4**	**±37.6**	**±1.9**	**±8.9**

[a] Fuc-BSA-FITC

[b] Background

[c] Percentage of the cells

[d] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.5B. Analytical data from Figure 47

pH 7.2

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)									
	Phosphate				Tris-HCl					
	Fuc[a]		BG[b]		Fuc		Fuc Ca^{++} Mg^{++}		BG	
	%[c]	FI[d]	%	FI	%	FI	%	FI	%	FI
	47.9	127.9	13.4	85.3	65.5	139.1	48.5	189.8	17.7	49.9
	69.1	108.2	13.9	40.2	82.9	134.3	81.6	143.7	13.0	37.1
	87.9	366.2	13.3	61.4	90.9	293.5	92.5	274.0	20.3	65.7
	91.0	381.7	8.0	77.0	90.6	312.0	93.1	253.6	13.7	48.9
Mean	**73.0**	**246.0**	**12.1**	**65.9±**	**82.4**	**219.7**	**78.9**	**215.2**	**16.1**	**50.4±**
±SEM	**±9.9**	**±74.0**	**±1.3**	**9.9**	**±5.9**	**±48.0**	**±10.4**	**±29.8**	**±1.7**	**5.8**

[a] Fuc-BSA-FITC

[b] Background

[c] Percentage of the cells

[d] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.6. Analytical data from Figure 49

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)							
	Fuc[a]		BG[b]		Fuc+ EDTA		BG+EDTA	
	%[c]	FI[d]	%	FI	%	FI	%	FI
	58.5	450.5	11.8	55.6	51.8	681.8	15.3	96.0
	48.6	542.6	4.0	108.0	46.2	761.0	6.1	202.0
	67.9	261.8	8.7	151.1	55.8	528.4	20.6	105.5
	54.4	319.7	2.9	402.2	48.0	609.5	9.1	204.8
	63.8	355.3	5.1	91.1	66.2	280.3	10.7	33.6
	52.8	435.4	2.6	40.9	60.4	323.2	17.2	54.9
Mean	**57.6**	**394.2**	**5.8**	**141.4**	**54.7**	**530.7**	**13.1**	**116.1**
±SEM	**±2.9**	**±41.2**	**±1.4**	**±54.5**	**±3.1**	**±79.1**	**±2.2**	**±29.5**

[a] Fuc-BSA-FITC

[b] Background

[c] Percentage of the cells

[d] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.7. Analytical analysis from Figure 51

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)											
Temperature	37°C				22°C				4°C			
	Fuc[a]		BG[b]		Fuc		BG		Fuc		BG	
	%[c]	FI[d]	%	FI	%	FI	%	FI	%	FI	%	FI
	74.8	173.0	7.2	33.5	49.9	127.2	10.6	36.6	44.1	126.9	6.8	42.1
	72.4	193.0	9.4	40.4	77.8	215.6	15.8	36.5	52.7	128.9	13.2	42.4
	82.3	384.3	9.0	42.3	62.0	232.9	9.7	46.0	52.0	431.7	18.7	57.5
	90.5	551.5	7.0	49.3	72.1	358.9	9.7	39.3	70.8	577.6	14.3	39.0
Mean	**80.9**	**325.6**	**8.1**	**41.3**	**65.9**	**233.6**	**10.9**	**39.6**	**54.9**	**396.2**	**13.2**	**45.2**
±SEM	**±3.7**	**±89.0**	**±0.6**	**±3.2**	**±6.3**	**±47.7**	**±1.3**	**±2.2**	**±5.6**	**±112.7**	**±2.4**	**±4.1**

[a] Fuc-BSA-FITC

[b] Background

[c] Percentage of the cells

[d] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.8. Analytical analysis from Figure 53

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)							
	Fuc[a]		BSA[b]		Lac[c]		BG[d]	
	%[e]	FI[f]	%	FI	%	FI	%	FI
	78.4	173.0	96.3	278.2	8.0	33.1	7.2	33.5
	72.4	193.0	98.1	427.3	6.8	93.6	9.4	40.4
	82.3	384.3	93.0	282.4	8.7	88.8	9.0	42.3
	90.5	551.5	95.3	264.1	13.0	46.2	7.0	49.3
Mean	**80.9**	**325.3**	**95.6**	**313.0**	**9.1**	**65.2**	**8.1**	**41.3**
±SEM	**±3.7**	**±89.0**	**±1.2**	**±38.2**	**±2.7**	**±15.1**	**±0.6**	**±3.2**

[a] Fuc-BSA-FITC

[b] BSA-FITC

[c] Lac-BSA-FITC

[d] Background

[e] Percentage of the cells

[f] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.9. Analyical data from Figure 55

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)											
	Fuc[a]										BG[b]	
	-		Fuc-BSA		Gal-BSA		L-Fucose		D-Galactose			
	%[c]	FI[d]	%	FI	%	FI	%	FI	%	FI	%	FI
	77.9	172.0	20.5	151.1	80.4	1036.4	76.9	172.7	78.3	151.4	1.8	46.4
	73.3	198.3	22.2	124.5	71.3	1093.3	77.0	131.6	74.4	161.9	2.3	241.5
	55.3	120.9	17.0	80.8	45.4	315.0	65.0	100.2	48.3	69.5	5.1	62.9
	56.2	131.1	16.6	91.2	63.9	411.7	59.0	100.0	67.1	119.8	4.1	36.3
Mean	**65.6**	**155.5**	**19.0**	**111.9**	**65.2**	**714.1**	**69.4**	**126.1**	**67.0**	**125.6**	**3.3**	**96.7**
±SEM	**±4.8**	**±18.0**	**±1.3**	**±16.0**	**±7.4**	**±203.0**	**±4.4**	**±17.2**	**±6.6**	**±20.7**	**±0.7**	**±48.5**

[a] Fuc-BSA-FITC

[b] Background

[c] Percentage of the cells

[d] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.10A. Analytical data from Figure 58

C. albicans **wild type (strain MEN)**

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)											
Time (h)	0				1				2			
	Fuc[a]		BG[b]		Fuc		BG		Fuc		BG	
	%[c]	FI[d]	%	FI	%	FI	%	FI	%	FI	%	FI
	64.4	283.5	-	-	78.0	332.0	-	-	82.0	337.8	-	-
	63.7	209.1	-	-	75.3	222.5	-	-	87.5	214.3	-	-
	13.3	194.8	0.07	21.1	61.6	194.1	0.4	33.0	78.8	193.8	0.3	29.2
	15.2	165.5	0.03	108.4	69.4	263.7	0.5	33.5	82.2	211.5	0.2	43.8
	48.2	347.4	0.3	32.7	76.2	578.6	0.5	30.2	87.3	340.5	0.8	41.0
	56.0	415.0	0.4	43.2	70.6	597.0	0.8	26.3	86.7	327.0	0.9	34.4
Mean	**43.4**	**269.2**	**0.2**	**51.3**	**71.8**	**364.6**	**0.5**	**30.7**	**84.0**	**270.8**	**0.5**	**37.1**
±SEM	**±9.5**	**±39.7**	**±0.05**	**±18.5**	**±2.4**	**±73.3**	**±0.05**	**±1.6**	**±1.4**	**±28.9**	**±0.1**	**±3.1**

[a] Fuc-BSA-FITC

[b] Background

[c] Percentage of the cells

[d] Mean fluorescence intensity (FCAU) of the cells

Appendix 6.10B. Analytical data from Figure 58

***C. albicans* germ tube-deficient mutant (strain MM2002)**

	Percentage of the cells within the boundaries and their mean fluorescence intensity (FCAU)											
Time (h)	0				1				2			
	Fuc[a]		BG[b]		Fuc		BG		Fuc		BG	
	%[c]	FI[d]	%	FI	%	FI	%	FI	%	FI	%	FI
	31.3	240.1	-	-	34.0	343.6	-	-	28.5	160.0	-	-
	23.2	187.6	-	-	29.2	162.3	-	-	27.5	118.2	-	-
	12.2	183.6	0.03	327.8	17.9	207.4	0.1	23.3	21.6	200.2	0.1	265.6
	13.3	239.2	0.1	894.3	21.7	304.1	0.07	26.3	27.2	337.0	0.1	27.6
	18.1	285.9	0.1	36.9	32.6	155.1	0.03	30.7	42.2	165.5	0.1	55.8
	17.7	171.0	0.1	26.9	46.1	259.0	0.2	44.4	39.9	163.1	0.1	24.4
Mean	**19.3**	**217.9**	**0.08**	**321.4**	**30.2**	**238.5**	**0.1**	**31.1**	**31.1**	**190.6**	**0.1**	**93.3**
±SEM	**±2.8**	**±18.1**	**±0.01**	**±203.7**	**±4.0**	**±31.1**	**±0.03**	**±4.6**	**±3.2**	**±31.1**	**±0.0**	**±57.9**

[a] Fuc-BSA-FITC

[b] Background

[c] Percentage of the cells within the boundaries

[d] Mean fluorescence intensity (FCAU) of the cells

Appendix 7. Transmission electron microscopy examinations of *C. albicans* : Labelling the cells with fibronectin coated-gold particles

Appendix 7.1. Buffers and Solutions for SDS-PAGE

Acrylamide (30% T, 2.67% C)

Acrylamide (29.2 g) and N', N'-bis-methylene-acrylamide (0.8 g) were made up to 100 ml DW. This solution was filtered and stored at 4°C.

1.5 M Tris buffer (pH 8.8)

1.5 M Tris buffer which was made up with tris base (18.15 g) in 100 ml DW. pH was adjusted with HCl.

0.5 M Tris buffer (pH 6.8)

0.5 M Tris buffer which was made up with tris base (6.0 g) in 100 ml DW. pH was adjusted with HCl.

10% Sodium dodecyl sulphate (SDS)

SDS (10 g) was dissolved in 100 ml DW.

10% Ammonium persulphate

Amonium persulphate (0.1 g) was dissolved in 1.0 ml DW and made up fresh.

0.05% Bromophenol blue

Bromophenol blue (0.005 g) was dissolved in 10 ml DW.

Solubilizing buffer

Solubilizing buffer was prepared containing the following stock solution: 0.5 M Tris-HCl pH 6.8 (1.0 ml), glycerol (0.8 ml), 10% SDS (1.6 ml), 2β-mercaptoethanol (0.4 ml), 0.05% (w/v) bromophenol blue (0.2 ml), DW (4.0 ml).

Reservoir buffer (pH 8.3)

Reservoir buffer was made up with tris base (3 g), glycine (14.4 g), SDS (1.0 g) in 1000 ml DW.

0.1% Coomassie brillant blue stain

Coomassie brillant blue stain was prepared from Coomassie blue (1.0 g), methanol (450 ml), acetic acid (100 ml) and DW (450 ml).

Destaining solution

Destaining solution was prepared with methanol (300 ml), acetic acid (100 ml) and DW (600 ml).

Separating gel preparation (7.5%)

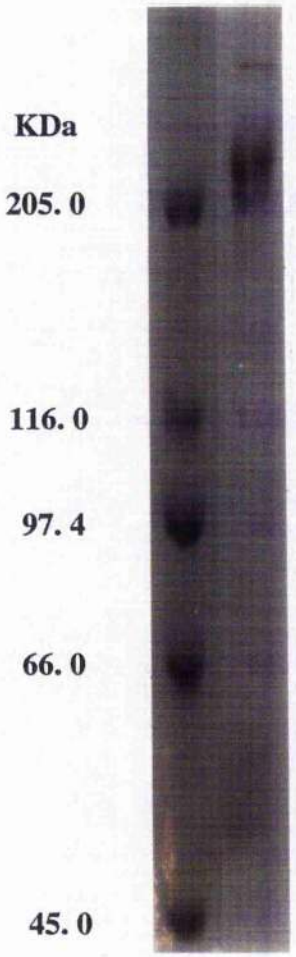
Separating gel was prepared with 1.5 M tris buffer pH 8.8 (17.5 ml), 10% SDS (0.7 ml), acrylamide\bio (17.5 ml), 10% ammonium persulphate (350 µl), TEMED (35 µl) and DW (70 ml).

Stacking gel preparation (4%)

Stacking gel preparation was prepared with 0.5 M tris buffer pH 6.8 (2.5 ml), 10% SDS (100 µl), acrylamide\bio (1.3 ml), 10% ammonium persulphate (50 µl), TEMED (10 µl) and DW (6.1 ml).

Running buffer Running buffer was prepared with tris (3 g), glycine (14.4 g) and SDS (1.0 g) in 1000 ml DW.

Appendix 7.2. The fibronectin was resolved on SDS-PAGE and loaded on to a 15mm thick, 4.5 w/v stacking gel and a 7.5 w/v polyacrylamide resolving gel . Electrophoresis was performed at 20 and 30 mA per gel respectively in a BRL (Bethesda Research Laboratory) gel apparatus. The gel was stained with Coomassie brillant blue. The standard molecular weight markers (in thousands) are on the left



Appendix 7.3. Serial dilutions of fibronectin in 2mM tris-HCl buffer containing 15 mM NaCl, and gold colloid to determine the minimum stabilizing concentration of fibronectin (values in microliters)

Fibronectin solution (250 µg ml^{-1})	2mM Tris HCl Buffer	Gold colloid	10% NaCl
50	0	250	50
30	20	250	50
20	30	250	50
10	40	250	50
0	50	250	0 (negative control)

1. 20 µl of fibronectin solution stabilized the 20 nm gold particles.
2. 350 ml solution total was used in the stability test.
3. 350 / 20 = 17.5 fold solution was added to bulk for coating the gold particle with fibronectin
4. 250 µl of fibronectin was used in this experiment
5. 17.5 x 250 = 4325 µl bulk solution mixed with gold particle and fibronectin
6. 4325 - 250 = 4125 µl gold solution was used.
7. 4125 µl gold solution was mixed in 250 µl fibronectin solution and stirred rapidly.
8. After 2 min stirring, secondary stabilizer (1% PEG pH 6.0) was added to give 0.02 % PEG.
9. The probe was centrifuged twice at 12.000 rpm.
10. The pellet was resuspended with Tris-HCl buffer containing 0.02% PEG and sodium azide. The concentration of probe was adjusted at $A_{520} = 1.0$.

Appendix 7.4. Processing of the cells labelled with fibronectin-coated gold particles (Au_{20}-Fn) for TEM

(For convenience, the steps are given as instructions)

1. Fix in 2.5 glutaraldehyde* in 0.1 M PBS for 2 h 20°C.
2. Store in the same buffer containing 2% sucrose at 4°C.
3. Rinse twice in Tris HCl buffer pH 7.4 for 10 min
4. Suspend in 1% Osmium tetroxide (OSO_4) for 1 h *.
5. Rinse three times in DW for 10 min each time.
6. Suspend in 0.5% Uranyl acetate for 30 min*.
7. Rinse once in DW for 2 min.
8. Dehydrate through an alcohol series 30, 50, 70, 90% for 5 min each followed by 2, 10 min changes in absolute alcohol the last change in dried absolute alcohol.
9. Rinse three times in epoxypropane (propylene oxide) for 5 min *
10. Suspend in 1 : 1 epoxypropane / Araldite and leave overnight on rotator in fume cupboard (caps off)*
11. Polymerize in fume hood oven at 60°C for 48 h

* Use fume cupboard and gloves

7. REFERENCES

AHEARN, D. G. (1978). Medically important yeasts. *Annual Review of Microbiology* **32,** 59-68.

ALAEI, S., LARCHER, C., EBENBICHLER, C., PRODINGER, W. M. , JANATOVA, J. and DIERICH, M. P. (1993). Isolation and biochemical characterization of the iC3b receptor of *Candida albicans. Infection and Immunity* **61,** 1395-1399.

AL-BASSAM T, POULAIN, D., GIUMMELLY, P. LEMATRE, J. and BONALY, R. (1985). Chemical and antigenic alterations of *Candida albicans* cell wall related to the action of amphotericin B sub-inhibitory doses. *Journal of Antimicrobial Chemotheraphy* **15,** 263-269.

ANAISSIE, E. J. (1992). Opportunistic mycoses in the immunocompromised host: experience at a cancer center and review. *Clinical Infectious Diseases* **14,** S43-S53.

ANNAIX, V., BOUCHARA, J.-P., TRONCHIN, G., SENET, J.-M. and ROBERT, R. (1990). Structures involved in the binding of human fibrinogen to *Candida albicans* germ tubes. *FEMS Microbiology Immunology* **64,** 147-154.

BARBER, C., EYLAN, E. HEIBER, R. (1973). Antibiotic activity of polysaccharide-protein complexes isolated from different *Candida* species. *Zentralblatt fuer Bakteriologie [A]* **223,** 542-548.

BARRETT-BEE, K., HAYES, Y., WILSON, R. G. and RYLEY, J. F. (1985). A comparison of phospholipase activity, cellular adherence and pathogenicity of yeasts. *Journal of General Microbiology* **131,** 1217-1221.

BENHAM, R. W. (1931). Certain monilias parasitic on man: their identification by morphology and by agglutination. *The Journal of Infectious Diseases* **49,** 183-215.

BENHAMOU, N. (1989). Preparation and application of lectin-gold complexes. In *Colloidal gold: Principles, methods, and applications.* ed. Hayat M. H. Vol 1, pp. 96-143. New York: Academic Press.

BLACKWELL, C. C., ALY, F. Z. , JAMES, V.S. and WEIR, D. M. (1990). Blood group status and oral carrige of yeast among patients with diabetes mellitus. *Diabetes Research* **12,** 101-104.

BODEY, G. P. (1993) *Candidiasis: pathogenesis, diagnosis and treatment* 2nd. New York: Raven Press, Ltd.

BOUALI, A., ROBERT, R., TRONCHIN, G. and SENET, J. M. (1986). Binding of human fibrinogen to *Candida albicans in vitro* : a preliminary study. *Journal of Medical and Veterinary Mycology* **24,** 345-348.

BOUALI, A., ROBERT, R., TRONCHIN, G. and SENET, J. M. (1987). Characterization of binding of human fibrinogen to the surface of germ-tubes and mycelium of *Candida albicans. Journal of General Microbiology* **133,** 545-551.

BOUCHARA, J. P. , TRONCHIN, G., ANNAIX, Y., ROBERT, R. and SENET, J. M. (1990). Laminin receptors on *Candida albicans* germ tubes. *Infection and Immunity* **58,** 48-54.

BOZZOLA, J. J. , METHA, R. J. , NISBET, L. J. and VALENTA, J. R. (1984). The effect of aculeacin A and papulacandin B on morphology and cell wall ultrastructure in *Candida albicans. Canadian Journal of Microbiology* **30,** 857-863.

BRASSART, D., WOLTZ, A., GOLLIARD, M. and NEESER, J.-R. (1991). *In vitro* inhibition of adhesion of *Candida albicans* clinical isolates to human buccal epithelial cells by Fuc α1-> 2Galβ-bearing complex carbodhyrates. *Infection and Immunity* **59,** 1605-1613.

BRAUN, P. C. and CALDERONE, R. A. (1978). Chitin synthesis in *Candida albicans* : comparison of yeast and hyphal forms. *Journal of Bacteriology* **135,** 1472-1477.

BUDTZ-JOERGENSEN, E. (1971). Proteolytic activity of *Candida* spp. as related to the pathogenesis of denture stomatitis. *Sabouraudia* **12,** 266-271.

BURFORD-MASON, A. P. , WEBER, J. C. P. and WILLOUGHBY, J. M. T. (1988). Oral carriage of *Candida albicans*, ABO blood group and secretor status in healthy subjects. *Journal of Medical and Veterinary Mycology* **26,** 49-56.

BUTRUS, S. I. and KLOTZ, S. A. (1986). Blocking *Candida* adherence to contact lenses. *Current Eye Research* **5,** 745-750.

CALDERONE, R. A., LEHRER, N. and SEGAL, E. (1984). Adherence of *Candida albicans* to buccal and vaginal epithelial cells: ultrastructural observations. *Canadian Journal of Microbiology* **30,** 1001-1007.

CALDERONE, R. A., CIHLAR, R. L. , LEE, D. D.-S., HOBERG, K. and SCHELD, W. M. (1985). Yeast adhesion in the pathogenesis of endocarditis due to *Candida albicans* : studies with adherence-negative mutants. *Journal of Infectious Diseases* **152,** 710-715.

CALDERONE, R. A. and SCHELD, W. M. (1987). Role of fibronectin in the pathogenesis of candidal infections. *Reviews of Infectious Diseases* **9,** S400- S403.

CALDERONE, R. A. and BRAUN, P. C. (1991). Adherence and receptor relationships of *Candida albicans. Microbiological Reviews* **55,** 1-20.

CALDERONE, R. A. (1993). Recognition between *Candida albicans* and host cells. *Trends in Microbiology* **1,** 55-58.

CALDERONE, R. A. and WADSWORTH, E. (1993). Adherence molecules of *Candida albicans. Journal of Microbiological Methods* **18,** 197-211.

CALDERONE, R. A., DIAMOND, R., SENET, J.-M., WARMINGTON, J., FILLER, S. and EDWARDS, J. E. (1994). Host cell-fungal cell interactions. *Journal of Medical and Veterinary Mycology* **32,** 151-168.

CAMERON, B. J. and DOUGLAS, L. J. (1996). Blood group glycolipids as epithelial cell receptors for *Candida albicans. Infection and Immunity* **64,** 891-896.

CANNON, R. D. (1986). Isolation of a mycelial mutant of *Candida albicans. Journal of General Microbiology* **132,** 2405-2407.

CASANOVA, M., LOPEZ-RIBOT, J. L. , MONTEAGUDO, C., LLOMBART-BOSH, A., SENTANDREU, R. and MARTINEZ, J. P. (1992). Identification of a 58-kilodalton cell surface fibronogen-binding mannoprotein from *Candida albicans. Infection and Immunity* **60,** 4221-4229.

CASSONE, A., SIMONETTI, N. and STRIPPOLI, V. (1973). Ultrastructural changes in the wall during germ-tube formation from blastospores of *Candida albicans*. *Journal of General Microbiology* **77,** 417-426.

CASSONE, A., KERRIDGE, D. and GALE, E. F. (1979). Ultrastructural changes in the cell wall of *Candida albicans* following cessation of growth and their possible relationship to the development of polyene resistance. *Journal of General Microbiology* **110,** 339-349.

CASTELLANI, A. (1912). Observation on the fungi found in tropical bronchomycosis. *Lancet* **1,** 13-15.

CENTENO, A., DAVIS, C. P. , COHEN, M.S. and WARREN, M. M. (1983). Modulation of *Candida albicans* attachment to human epithelial cells by bacteria and carbohydrates. *Infection and Immunity* **39,** 1354-1360.

CHATTAWAY, F. W. , HOLMES, M. R. and BARLOW, A. J. E. (1968). Cell wall composition of the mycelial and blastrospore forms of *Candida albicans*. *Journal of General Microbiology* **51,** 367-376.

CHIEW, Y. Y. , SHEPHERD, M. G. and SULLIVAN, P. A. (1980). Regulation of chitin synthesis during germ-tube formation in *Candida albicans*. *Archives of Microbiology* **125,** 97-104.

COLEMAN, D. C., BENNETT, D. E., SULLIVAN, D. J. , GALLAGHER, P. J. , HENMAN, M. C., SHANLEY, D. B. and RUSSELL, R. J. (1993). Oral *Candida* in HIV infection and AIDS: new perspectives / new approaches. *Critical Reviews in Microbiology* **19,** 61-82.

COLLINS-LECH, C., KALBFLEISCH, J. H. , FRANSON, T. R. and SOHNLE, P. G. (1984). Inhibition by sugars of *Candida albicans* adherence to human buccal mucosal cells and corneocytes *in vitro*. *Infection and Immunity* **46,** 831-834.

CORMANE, R. H. and GOSLINGS, W. R. O. (1963). Factors influencing the growth of *Candida albicans* (*in vivo* and *in vitro* studies) *Sabouraudia* **3,** 52-63.

CRITCHLEY, I. A. and DOUGLAS, L. J. (1985). Differential adhesion of pathogenic *Candida* species to epithelial and inert surfaces. *FEMS Microbiology Letters* **28,** 199-203.

CRITCHLEY, I. A. (1986). Mechanism of Adherence of *Candida albicans* to Epithelial cells. Ph. D. Thesis. University of Glasgow, Scotland.

CRITCHLEY, I. A. and DOUGLAS, L. J. (1987a). Isolation and partial characterization of an adhesin from *Candida albicans*. *Journal of General Microbiology* **133,** 629-636.

CRITCHLEY, I. A. and DOUGLAS, L. J. (1987b). Role of glycosides as epithelial cell receptors for *Candida albicans*. *Journal of General Microbiology* **133,** 637-643.

CUTLER, J. E., FRIEDMAN, L. and MILNER, K. C. (1972). Biological and chemical characterization of toxic substances from *Candida albicans*. *Infection and Immunity* **6,** 616-627.

CUTLER, J. E. (1991). Putative virulence factors of *Candida albicans*. *Annual Review of Microbiology* **45,** 187-218.

DABROWA, N., LANDAU, J. W. and NEWCOMER, V. D. (1968). Generation time of *Candida albicans* in synchronized and nonsynchronized cultures. *Sabouraudia* **6,** 51-56.

DABROWA, N., HOWARD, D. H. , LANDAU, J. W. and SHECHTER, Y. (1970). Synthesis of nucleic acids and proteins in the dimorphic forms of *Candida albicans*. *Sabouraudia* **8,** 163-169.

DAVENPORT, J. C. (1970). The oral distribution of *Candida* in denture stomatitis. *British Dental Journal* **129,** 151-156.

DEPIERREUX, C., KANG, H.-C., GUERIN, B., MONSIGNY, M. and DELMOTTE, F. (1991). Characterization of *Agrobacterium tumefaciens* lectin. *Glycobiology* **1,** 643-649.

DE MEY, J. R. (1986). The preparation and use of gold probes. In *Immunocytochemistry : Modern methods and applications* ed. Polak , J. M. and Van. Noorden, S. 2nd ed. pp. 115-137. Wright PSG: Bristol.

DERESINSKI, S. C., PAPAGIANIS, D. and STEVENS, D. A. (1979). Association of ABO blood groups and outcome of coccidiodal infection. *Sabouraudia* **17,** 261-267.

DOUGLAS, L. J. , HOUSTON, J. G. and MCCOURTIE, J. (1981). Adherence of *Candida albicans* to human buccal epithelial cells after growth on different carbon sources. *FEMS Microbiology Letters* **12,** 241-243.

DOUGLAS, L. J. (1987a). Adhesion of *Candida* species to epithelial surfaces. *CRC Critical Reviews in Microbiology* **15,** 27-43.

DOUGLAS, L. J. (1987b). Adhesion to surfaces. In. *The Yeast* ed. Rose, A. H. and Harrison, J. S. Vol. 2, pp. 239-280. London: Academic Press.

DOUGLAS, L. J. (1991). Adhesion of *Candida albicans* to host surfaces. In *Proceeding of the FEMS Symposium on Candida and Candida mycosis* ed. Tumbay, E. , Seeliger, H. P. R. and Ang, O. Vol. 50, pp. 43-48. New York: Plenum Press.

DOUGLAS, L. J. (1992). Mannoprotein adhesins of *Candida albicans*. In *New Strategies in Fungal Disease* ed. Bennett, J. E. , Hay, R. J. and Peterson, P. K. pp. 34-53. London: Churchill Livingstone.

DUBOIS, M., GILLES, K. A., HAMILTON, J. K. , REBERS, P. A. and SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28,** 350-356.

DUPONT, B., GRAYBILL, J. R. , ARMSTRONG, D., LAROCHE, R., E., T. J. and WHEAT, L. J. (1992). Fungal infections in AIDS patients. *Journal of Medical and Veterinary Mycology* **30,** 19-28.

EDWARDS, J. E., MAYER, C. L. , FILLER, S. G. , WADSWORTH, E. and CALDERONE, R. A. (1992). Cell extracts of *Candida albicans* block adherence of the organisms to endothelial cells. *Infection and Immunity* **60,** 3087-3091.

EPSTEIN, J. B., KIMURA, L. H., MENARD, T.W., TRUELOVE, E. L. AND PERSALL, N. N. (1982). Effects of specific antibodies on the interaction between the fungus *Candida albicans* and human oral mucosa *Archives of Oral Biology* **27,** 469-474.

FALK, P., ROTH, K. A. , BOREN, T., WESTBLOM, T.U., GORDON, J. I., NORMARK, S. (1993). An *in vitro* adherence assay reveals that *Helicobacteri pylori* exhibits cell lineage-specific tropism in the human gastric epithelium. *Proceedings of the National Academy of Sciences of the United States of America,* **90,** 2035-2039.

FARRELL, S. M., HAWKINS, D. F. and RYDER, T. A. (1983). Scanning electron microscope study of *Candida albicans* invasion of cultured human cervical epithelial cells. *Sabouraudia* **21,** 251-254.

FUKAYAMA, M. and CALDERONE, R. A. (1991). Adherence of cell surface mutants of *Candida albicans* to buccal epithelial cells and analyses of the cell surface proteins of the mutants. *Infection and Immunity* **59,** 1341-1345.

GEOGHEGAN, W. D. and ACKERMAN, G. A. (1977). Adsorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ aglutinin and goat anti-human immunoglobulin G on cell surfaces at the electron microscopic level: a new method, theory and application. *The Journal of Histochemistry and Cytochemistry* **25,** 1187-1200.

GHANNOUM, M. A. and ABU-ELTEEN, K. (1986). Correlative relationship between proteinase production, adherence and pathogenicity of various strains of *Candida albicans*. *Journal of Medical and Veterinary Mycology* **24,** 407-413.

GHANNOUM, M. A., BURNS, G. R. , ABU-ELTEEN, K.. and RADWAN, S. S. (1986). Experimental evidence for the role of lipids in adherence of *Candida* spp. to human buccal epithelial cells. *Infection and Immunity* **54,** 189-193.

GHANNOUM, M. A., ABU-ELTEEN, K. and RADWAN, S.S. (1987). Blocking adherence of *Candida albicans* to buccal epithelial cells by yeast glycolipids, yeast wall lipids and lipids from epithelial cells. Mykosen 30, 371-378.

GHANNOUM, M. A. and ABU-ELTEEN, K. (1991). Adherence of Candida albicans : influencing factors and mechanism (s). In *Candida albicans Cellular and Molecullar Biology* ed. Prassad, R. pp.144-163. Berlin Heidelberg: Spinger-Verlag.

GHANNOUM, M. A., ABU-ELTEEN, K. and RADWAN, S. S. (1987). Blocking adherence of *Candida albicans* to buccal epithelial cells by yeast glycolipids, yeast wall lipids and lipids from epithelial cells. *Mycosen* **30,** 371-378.

GILMORE, B. and HOSTETTER, M. K. (1986). Inducible binding site for iC3b on virulent *Candida albicans*. *Clinical Research* **34,** 518A.

GILMORE, B. J. , RETSINAS, E. M. , LORENZ, J.S. and HOSTETTER, M. K. (1988). An iC3b receptor on *Candida albicans* : structure, function and correlates for pathogenicity. *The Journal of Infectious Diseases* **157,** 38-46.

GOPAL, P. K. , SHEPHERD, M. G. and SULLIVAN, P. A. (1984). Analysis of wall glucans from yeast, hyphal and germ-tube forming cells of *Candida albicans*. *Journal of General Microbiology* **130,** 3295-3301.

GREEN, L. PETERSEN, B., STEIMEL, L., HAEBER, P. AND CURRENT, W. (1994). Rapid determination of antifungal activity by flow cytometry *Journal of Clinical Microbiology* **32,** 1088-1091.

GUSTAFSON, K.S., VERCELLOTTI, G. M. , BENDEL, C. M. and HOSTETTER, M. K. (1991). Molecular mimicry in *Candida albicans*. Role of an integrin analog in adhesion of the yeast to human endothelium. *Journal of Clinical Investigations* **87,** 1896-1902.

HAMILTON-MILLER, J. M. T. (1973). Chemistry and biology of the polyene macrolide antibiotics. *Bacteriological Reviews* **37,** 166-196.

HASENCLEVER, H. F. and MITCHELL, W. O. (1961). Antigenic studies of *Candida* I. Observation of two antigenic groups in *Candida albicans*. *Journal of Bacteriology* **82,** 570-573.

HASENCLEVER, H. F. and MITCHELL, W. O. (1963). Endotoxin-induced tolerance to toxic manifestations of *Candida albicans*. *Journal of Bacteriology* **85,** 1088-1093.

HAWSER, S. P. and DOUGLAS, L. J. (1994). Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. *Infection and Immunity* **62,** 915-921.

HAZEN , B. W. and HAZEN, K. C. (1988). Dynamic expression of cell surface hydrophobicity during yeast cell growth and before germ tube formation of *Candida albicans*. *Infection and Immunity* **57,** 167-172.

HAZEN, K. C. (1989). Isolation of hydrophobic and hydrophilic mutants of *Candida albicans*. *Infection and Immunity* **57,** 1894-1900.

HECTOR, R. F., BRAUN, P.C., HART, J. T. and KAMARCK, M. E. (1990). The use of flow cytomerty to monitor chitin synthesis in regenerating protoplasts of *Candida albicans*. *Journal of Medical and Veterinary Mycology* **28** 51-57.

HILENSKI, L. L. , NAIDER, F. and BECKER, J. M. (1986). Polyoxin D inhibits colloidal gold-wheat germ tube agglutinin labelling of chitin in dimorphic forms of *Candida albicans*. *Journal of General Microbiology* **132,** 1441-1451.

HILL, D. W. and GERBHARDT, L. P. (1956). Morphological transformation of *Candida albicans* in tissues of mice. *Proceedings of the Society of Experimental Biology and Medicine* **92,** 640-644 .

HOLMES, A. R., CANNON, R.D. and SHEPHERD, M. G. (1991). Effect of calcium ions uptake on *Candida albicans*. *FEMS Microbiology Letters* **77,** 187-193.

HORISBERGER, M. and ROSSET, J. (1977). Colloidal gold, a useful marker for transmission and scanning electron microscopy. *The Journal of Histochemistry and Cytochemistry* **25,** 295-305.

HOSTETTER, M. K. (1994a). Adhesins and ligands involved in the interaction of *Candida* spp. with epithelial and endothelial surfaces. *Clinical Microbiology Reviews* **7,** 29-42.

HOSTETTER, M. K. (1994b). Interactions of *Candida albicans* with eukaryotic cells. *American Society of Microbiology News* **60,** 370-374.

HOWLETT, J. A. and SQUIER, C. A. (1980). *Candida albicans* ultrastructure: colonization and invasion of oral epithelium. *Infection and Immunity* **29,** 252-260.

HUBBARD, M. J. , SULLIVAN, P. A. and SHEPHERD, M. G. (1985). Morphological studies of *N*- acetylglucosamine induced germ tube formation. *Canadian Journal of Microbiology* **31,** 696-701.

HUBBARD, M. J. , MARKIE, D. AND POULTER , R. T. M. (1986). Isolation and morphological characterization of a mycelial mutant of *C. albicans. Journal of Bacteriology* **165,** 61-65.

JAKAP, E., PAULSSON, M., ASCENCIO, F. and LJUNGH, A. (1993). Expression of vitronectin and fibronectin binding by *Candida albicans* yeast cells. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* **101,** 187-193.

JIMENEZ-LUCHO, V., GINSBURG, V. and KRIVAN, H. C. (1990). *Cryptococcus neoformans, Candida albicans,* and other fungi bind specially to the glycosphingolipid lactosylceramide (Galβ1-4 Glcβ1-1Cer), a possible adhesion receptor for yeasts. *Infection and Immunity* **58,** 2085-2090.

JOHNSON, E.M., RICHARDSON, M. D. and WARNOCK, D. W. (1983). Effect of imidazole antifungals on the development of germ tubes by strains of *Candida albicans. Journal of Antimicrobial Chemotherapy* **12,** 303-316.

JONES, G. W. and FRETER, R. (1976). Adhesive properties in *Vibrio cholerae* : nature of the interaction with isolated rabbit brush border membranes and human erythrocytes. *Infection and Immunity* **14,** 240-245.

KALO, A., SEGAL, E., SAHAR, E. and DAYAN, D. (1988). Interaction of *Candida albicans* with genital mucosal surfaces: involvement of fibronectin in adherence. *The Journal of Infectious Diseases* **157,** 1253-1256.

KANBE, T., HAN, Y., REDGRAVE, B., RIESSELMAN, M. H. and CUTLER, J. E. (1993). Evidence that mannans of *Candida albicans* are responsible for adherence of yeast forms to spleen and lymph node tissue. *Infection and Immunity* **61,** 2578-2584.

KARABINIS, A., HILL, C., LECLERCQ, B., TANCREDE, C., BAUME, D. and ANDREMONT, A. (1988). Risk factors for candidemia in cancer patients: A case-control study. *Journal of Clinical Microbiology* **26,** 429-432.

KASHBUR, I. M. , AYLIFFE, G. A. J. and GEORGE, R. H. (1980). The survival of *Candida albicans* in moist and dry environments. *Journal of Hospital Infection* **1,** 349- 356.

KEARNS, M. J. , DAVIES, P. and SMITH, H. (1983). Variability of the adherence of *Candida albicans* strains to human buccal epithelial cells: inconsistency of differences between strains related to virulence. *Sabouraudia* **21,** 93-98.

KENNEDY, M. J. (1981). Inhibition of *Candida albicans* by anaerobic oral flora of mice *in vitro*. *Sabouraudia* **19,** 205-208.

KENNEDY, M. J. and VOLZ, P, A. (1985). Effect of various antibiotics on gastrointestinal colonization and dissemination by *Candida albicans*. *Journal of Medical and Veterinary Mycology* **23,** 265-273.

KENNEDY, M. J, VOLZ, P. A., EDWARDS, C. A. and YANCEY, R. J. (1987). Mechanisms of association of *Candida albicans* with intestinal mucosa. *Journal of Medical Microbiology* **24,** 333-341.

KENNEDY, M. J. and SANDIN, R. L. (1988). Influence of growth conditions on *Candida albicans* adhesion hydrophobicity and cell wall ultrastructure. *Journal of Medical and Veterinary Mycology* **26,** 79-92.

KENNEDY, M. J., ROGERS, A. L., HANSELMEN, L. R., SOLL, D. R. and YANCEY, R. J. (1988) Variation in adhesion and cell surface hydrophobicity in *Candida albicans* white and opaque phenotypes. *Mycopathologia* **102,** 149-156.

KENNEDY, M. J. , ROGERS, A. L. and YANCEY, R. J. (1989). Enviromental alteration and phenotypic regulation of *Candida albicans* adhesion to plastic. *Infection and Immunity* **57,** 3876-3881.

KENNEDY, M. J. , CALDERONE, R. A., CUTLER, J. E., KANABE, T., RIESSELMAN, M. H. , ROBERT, R., SENET, J.-M., ANNAIX, V., BOUALI, A., MAHAZA, C., TRONCHIN, G., BOUCHARA, J.-P., MIEGEVILLE, M., MAROT-LEBLOND, A. and SEGAL, E. (1992). Molecular basis of *Candida albicans* adhesion. *Journal of Medical and Veterinary Mycology* **30,** 95-122.

KIMURA, L. H. and PEARSALL, N. N. (1978). Adherence of *Candida albicans* to human buccal epithelial cells. *Infection and Immunity* **21,** 64-68.

KIMURA, L. H. and PEARSALL, N. N. (1980). Relationship between germination of *Candida abicans* and increased adherence to human buccal epithelial cells. *Infection and Immunity* **28,** 464-468.

KING, R. D., LEE, J. C. and MORRIS, A. L. (1980). Adherence of *Candida albicans* and other *Candida* species to mucosal epithelial cells. *Infection and Immunity* **27,** 667-674.

KLOTZ, S. A., DRUTZ, D. J. , HARRISON, J. L. and HUPPERT, M. (1983). Adherence and penetration of vascular endothelium by *Candida* yeasts. *Infection and Immunity* **42,** 374-384.

KLOTZ, S. A., DRUTZ, D. J. and ZAJIC, J. E. (1985). Factors governing adherence of *Candida* species to plastic surfaces. *Infection and Immunity* **50,** 97-101.

KLOTZ, S. A. (1987). The adherence of *Candida* yeasts to human and bovine vascular endothelium and subendothelial extracellular matrix. *FEMS Microbiology Letters* **48,** 201-205.

KLOTZ, S. A. and PENN, R.L. (1987). Multiple mechanisms may contribute to the adherence of *Candida* yeasts to living cells. *Current Microbiology* **16,** 119-122.

KLOTZ, S. A. and MACA, R. D. (1988). Endothelial cell contraction increases *Candida* adherence to exposed extracellular matrix. *Infection and Immunity* **56,** 2495-2498.

KLOTZ, S. A., HARRISON, J. L. and MISRA, R. P. (1989). Aggregated platelets enhance adherence of *Candida* yeast to endothelium. *The Journal of Infectious Diseases* **160,** 669-677.

KLOTZ, S. A. and SMITH, R. L. (1990). *Candida albicans* adherence to subendothelial extracellular matrix and matrix components is inhibited by arginine-glycine-aspartic acid peptides. *Clinical Research* **38,** 13A.

KLOTZ, S. A. and SMITH, R. L. (1991). A fibronectin receptor on *Candida albicans* mediates adherence of the fungus to extracellular matrix. *The Journal of Infectious Diseases* **163,** 604-610.

KLOTZ, S. A., SMITH, R. L. and STEWART, B. W. (1992). Effect of an arginine-glycine-aspartic acid-containing peptide on hematogenous candidal infectious in rabbits. *Antimicrobial Agents and Chemotherapy* **36,** 132-136.

KLOTZ, S. A., RUTTEN, M. J. , SMITH, S. L. , BABCOCK, S. R. and CUNNINGHAM, M. D. (1993). Adherence of *Candida albicans* to immobilized extracellular matrix proteins is mediated by calcium-dependent surface glycoproteins. *Microbial Pathogenesis* **14,** 133-147.

KLOTZ, S. A., HEIN, R. C., SMITH, R. L. and ROUSE, J. B. (1994). The fibronectin adhesin of *Candida albicans*. *Infection and Immunity* **62,** 4679-4681.

KOCH, Y. and KOCH, H. A. (1981). Zur epidemiologie der candidosen des mund-pharynx-raumes. *Mykosen* **24,** 197-202.

KOZINN, P. J. , TASCHDJIAN, C. L. and BURCHALL, J. J. (1960). Transmission of ^{32}P-labelled *Candida albicans* to newborn mice at birth. *American Journal of Diseases of Children* **99,** 31-34.

KUUSELA, P., VARTIO,T., VUENTO, M., and MYHRE, E. B. (1984). Binding sites for streptococci and staphylococci in fibronectin. *Infection and Immunity* **45,** 433-436.

KWON-CHUNG, K. J. , LEHMAN, D., GOOD, C. and MAGEE, P. T. (1985). Genetic evidence for role of extracellular proteinase in virulence of *Candida albicans*. *Infection and Immunity* **49,** 571-575.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. *Nature* **227,** 680-685.

LATGE, J. P. , MONSIGNY, M. and PREVOST, M.-C. (1988). Visualization of exocellular lectins in the entomopathogenic fungus *Conidiobolus obscurus*. *The Journal of Histochemistry and Cytochemistry* **36,** 1419-1424.

LEE, K. L. , BUCKLEY, H. R. and CAMPBELL, C. C. (1975). An amino acid liquid synthetic medium for development of mycelial and yeast forms of *Candida albicans*. *Sabouraudia* **13,** 148-153.

LEE, J. C. and KING, R. D. (1983). Characterization of *Candida albicans* adherence to human vaginal epithelial cells *in vitro*. *Infection and Immunity* **41,** 1024-1030.

LEHRER, N., SEGAL, E., CIHLAR, R. L. and CALDERONE, R. A. (1986). Pathogenesis of vaginal candidiasis: studies with a mutant which has reduced ability to adhere *in vitro*. *Journal of Medical and Veterinary Mycology* **24,** 127-131.

LEHRER, N., SEGAL, E., LIS, H. and GOV, Y. (1988). Effect of *Candida albicans* cell wall components on the adhesion of the fungus to human and vaginal mucosa. *Mycopathologia* **102,** 115-121.

LI, R.-K. and CUTLER, J. E. (1993). Chemical definition of an epitope/ adhesin molecule on *Candida albicans*. *Journal of Biological Chemistry* **268,** 18293-18299.

LILJEMARK, W. F. and GIBBONS, R. J. (1973). Suppression of *Candida albicans* by human oral streptococci in gnotobiotic mice. *Infection and Immunity* **8,** 846-849.

LIMPER, A. H. AND STANDING, J. E. (1994). Vitronectin interacts with *Candida albicans* and augments organism attachment to the NR8383 macrophage cell line. *Immunology Letters* **42,** 134-144.

LOPEZ-RIBOT, J. L. , CASANOVA, M., MARTINEZ, J. P. and SENTANDREU, R. (1991). Characterization of cell wall proteins of yeast and hydrophobic mycelial cells of *Candida albicans*. *Infection and Immunity* **59,** 2324-2332.

LOPEZ-RIBOT, J. L. AND CHAFFIN, W. L. (1994). Binding of the extracellular matrix component entactin to *Candida albicans*. *Infection and Immunity* **62,** 4564-4571.

LOPEZ-RIBOT, J. L. , CASANOVA, M., MONTEAGUDO, C., SEPULVEDA, P. and MARTINEZ, J. P. (1994). Evidence for the presence of a high-affinity laminin receptor-like molecule on the surface of *Candida albicans* yeast cells. *Infection and Immunity* **62,** 742-746.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. L. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemisty* **193,** 265-275.

LYNCH, M. E., KUKURUGA, M., NAKEFF, A., FIDEL, P. L. and SOBEL, J. D. (1993). Flow cytometric analysis of germ tube formation in *Candida albicans*. *Journal of Medical and Veterinary Mycology* **31,** 367-376.

MACDONALD, F. and ODDS, F. C. (1980). Inducible proteinase of *Candida albicans* in diagnostic serology and in the pathogenesis of systemic candidosis. *Journal of Medical Microbiology* **13,** 423-435.

MACDONALD, F. and ODDS, F. C. (1983). Virulence for mice of a proteinase-secreting strain of *Candida albicans* and a proteinase-deficient mutant. *Journal of General Microbiology* **129,** 431-438.

MACKENZIE, D. W. R. (1964). Morphogenesis of *Candida albicans in vivo*. *Sabouraudia* **3,** 225-232.

MAHAZA, C., ROBERT, R. and SENET, J. M. (1991). *Candida albicans* -platelet interactions: molecules involved in the adherence. In *Proceeding of the FEMS Symposium on Candida and Candida mycosis* ed. Tumbay, E. , Seeliger, H. P. R. and Ang, O. Vol. 50, pp. 137-141. New York: Plenum Press.

MAISH, P. A. and CALDERONE, R. (1980). Adherence of *Candida albicans* to fibrin-platelet matrix formed *in vitro*. *Infection and Immunity* **27,** 650-656.

MAISH, P. A. and CALDERONE, R. (1981). Role of surface mannan in the adherence of *Candida albicans* to fibrin-platelet clots formed *in vitro*. *Infection and Immunity* **32,** 92-97.

MARRIE, T. J. and COSTERTON, J. W. (1981). The ultrastructure of *Candida albicans* infections *Canadian Journal of Microbiology* **27,** 1156-1164.

MARTIN, M. V. CRAIG, G. T. LAMB, D. J. (1984) An investigation of the role of true hypha production in the pathogenesis of experimental oral candidosis. *Journal of Medical and Veterinary Mycology* **22,** 471-476.

MARTINEZ, J. P. , LOPEZ-RIBOT, J. L. and CHAFFIN, W. L. (1994). Heterogenous surface distribution of the fibrinogen-binding protein on *Candida albicans*. *Infection and Immunity* **62,** 709-712.

MASY, C. L. , HENQUINET, A. and MESTDAGH, M. M. (1991). Fluorescence study of lectinlike receptors involved in the flocculation of the yeast *Saccharomyces cerevisiae*. *Canadian Journal of Microbiology* **38,** 405-409.

MAY, S. J. , BLACKWELL, C. C. and WEIR, D. M. (1989). Lewis^a blood group antigen of non-secretors: a receptor for *Candida* blastospores. *FEMS Microbiology and Immunology* **47,** 407-410.

MAYER, C. L. , FILLER, S. G. and EDWARDS, J. E. (1992). *Candida albicans* adherence to endothelial cells. *Microvascular Research* **43,** 218-226.

MCCOURTIE, J. and DOUGLAS, L. J. (1981). Relationship between cell surface composition of *Candida albicans* and adherence to acrylic after growth on different carbon sources. *Infection and Immunity* **32,** 1234-1241.

MCCOURTIE, J. and DOUGLAS, L. J. (1984). Relationship between cell surface composition, adherence, and virulence of *Candida albicans*. *Infection and Immunity* **45,** 6-12.

MCCOURTIE, J. and DOUGLAS, L. J. (1985). Extracellular polymer of *Candida albicans* : isolation, analysis and role in adhesion. *Journal of General Microbiology* **131,** 495-503.

MEHENTEE, J. F. and HAY, R. J. (1989). *In vitro* adherence of *Candida albicans* strains to murine gastrointestinal mucosal cells and explants and the role of environmental pH. *Journal of General Microbiology* **135,** 12181-2188.

MERSON-DAVIES, L. A. and ODDS, F.C. (1989). A morphology index for characterization of cell shape in *Candida albicans*. *Journal of General Microbiology* **135,** 3143-3152.

MEYER, S. A., AHEARN, D. G. and YARROW, D. G. (1984). The genus *Candida* Berkhout. In *The yeast: a taxonomic study* ed. Kreger-van Rij N. J. W. pp. 585-844. Elsevier Science Publishers B. V. :Amsterdam.

MIDOUX, P., ROCHE, A.-C. and MONSIGNY, M. (1987). Quantitation of the binding, uptake, and degradation of fluoresceinylated neoglycoproteins by flow cytometry. *Cytometry* **8,** 327-334.

MINAGI, S., MIKAYE, Y., INAGAKI, K., TSURU, H. and SUGINATA, H. (1985). Hydrophobic interactions in *Candida albicans* and *Candida tropicalis* adherence to various denture base resin materials. *Infection and Immunity* **47,** 11-14.

MISHRA, P. and PRASAD, R. (1991). Lipids of *Candida albicans*. In *Candida albicans Cellular and Molecullar Biology*, ed. Prassad, R. pp.144-163. Berlin Heidelberg: Spinger-Verlag.

MIYAKAWA, Y., KAGAYA, K., FUKAZAWA, Y. and SOE, G. (1986). Production and characterization of agglutinating monoclonal antibodies against predominant antigenic factors for *Candida albicans*. *Journal of Clinical Microbiology* **23,** 881-886.

MIYAKAWA, Y., KURIBAYASHI, T., KAGAYA, K., SUZUKI, M., NAKASE, T. and FUKAZAWA, Y. (1992). Role of specific determinants in mannan of *Candida albicans* serotype A in adherence to human buccal epithelial cells. *Infection and Immunity* **60,** 2493-2499.

MIYAKE, Y., FIJITA, Y., MINAGI, S. and SUGINAKA, H. (1986). Surface hydrophobicity and adherence of *Candida* to acrylic surfaces. *Microbios* **46,** 7-14.

MOLLICONE, R., BARA, J., LE-PENDU, J and ORIOL, R. (1985). Immunohistologic pattern of type 1 (Le^a, Le^b) and type 2 (X, Y, H) blood group-related antigens in the human pyloric and duodenal mucosae. *Laboratory Investigation* **53,** 219-227.

MONSIGNY, M., ROCHE, A.-C. and MIDOUX, P. (1984). Uptake of neoglycoproteins via membrane lectin(s). of L1210 cells evidenced by quantitative flow cytofluorometry and drug targeting. *Biology of the Cell* **51,** 187-196.

MONSIGNY, M., ROCHE, A.-C., MIDOUX, P. and MAYER, R. (1994). Glycoconjugates as carriers for specific delivery of therapeutic drugs and genes. *Advanced Drug Delivery Reviews* **14,** 1-24.

MUERKOESTER, G. C., KOMOROWSKI, R. A. and FARMER, S. G. (1979). A comparison of hyphal growth of *Candida albicans* in six liquid media. *Sabouraudia* **17,** 55-64.

NEGRE, E., VOGEL, T., LEVANON, A., GUY, R., WALSH, T. J. and ROBERTS, D. D. (1994). The collagen binding domain of fibronectin contains a high affinity binding site for *Candida albicans*. *Journal of Biological Chemistry* **269,** 22039-22045.

NELSON, R. D., SHIBATA, N., PODZORSKI, R. P. and HERRON, M. J. (1991). *Candida* manan: chemistry, suppression of cell-mediated immunity, and possible mechanisms of action. *Clinical Microbiology Reviews* **4,** 1-19.

NOTARIO, V. (1982). β-glucanases from *Candida albicans* : purification, characterization and the nature of their attachment to cell wall components. *Journal of General Microbiology* **128,** 747-759.

ODDS, F. C. (1985). Morphogenesis in *Candida albicans*. *CRC Critical Reviews in Microbiology* **12,** 45-93.

ODDS, F. C. (1988). *Candida and Candidosis*. London: Bailliere Tindall.

ODDS, F. (1994). *Candida* species and virulence. *American Society of Microbiology News* **6,** 313-318.

OFEK, I., MIRELMAN, D. and SHARON, N. (1977). Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. *Nature* **265,** 623-625.

OFEK, I. and BEACHEY,. E. H. (1978). Mannose binding and epithelial cell adherence of *Escherichia coli*. *Infection and Immunity* **22,** 247-254.

OLLERT, M. W. , WADSWORTH, E. and CALDERONE, R. A. (1990). Reduced expression of the functionally active complement receptor for iC3b but not for C3d on an avirulent mutant of *Candida albicans*. *Infection and Immunity* **58,** 909-913.

OLLERT, M. W. , SOHNCHEN, R., KORTING, H. C., OLLERT, U., BRAUTIGAM, S. and BRAUTIGAM, W. (1993). Mechanisms of adherence of *Candida albicans* to cultured human epidermal keratinocytes. *Infection and Immunity* **61,** 4560-4568.

OLSEN, A. JONSSON, A. and NORMARK, S. (1989) Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. *Nature* **338,** 652-655.

OLSEN, I. (1990). Oral adhesion of yeasts. *Acta Odontologia Scandinavica* **49,** 45-53.

PAGE, S. and ODDS, F. C. (1988). Binding of plasma proteins to *Candida* species *in vitro*. *Journal of General Microbiology* **134,** 2693-2702.

PENDRAK, M. L. and KLOTZ, S. A. (1995) Adherence of *Candida albicans* to host cells. *FEMS Microbiology Letters* **129,** 103-114.

PENN, C. and KLOTZ, S. A. (1994). Binding of plasma fibronectin to *Candida albicans* occurs through the cell binding domain. *Microbial Pathogenesis* **17,** 387-393.

PERSI, M. A., BURNHAM, J. C. and DUHRING, J. L. (1985). Effect of carbon dioxide and pH on adhesion of *Candida albicans* to vaginal epithelial cells. *Infection and Immunity* **50,** 82-90.

PESCIOTTA PETERS, D. M. and MOSHER, D. F. (1987). Assembly and alignment of fibronectin-coated gold beads into fibrils by human skin fibroblasts. *Scanning Microscopy,* **1,** 141-146.

PIKE, I.H., EVANS, E.G.V. and CARNEY, J. A. (1991). Mannan estimation as a measure of the growth of *Candida albicans. Journal of Medical and Veterinary Mycology* **29,** 83-91.

POLACHECK, I., ANTMAN, A., BARTH, I., SAGI, E. and GILOH, H. (1995). Adherence of *Candida albicans* to epithelial cells: studies using fluorescently labelled yeasts and flow cytometry. *Microbiology* **141,** 1523-1533.

POPE, L. M. and COLE, G. T. (1981). Scanning electron microscope studies of adherence of *C. albicans* to the gastrointestinal tract of infant mice. *Scanning Electron Microscopy* **3,** 73-80.

PORE, R. S. (1990). Antibiotic susceptibility testing of *Candida albicans* by flow cytometry. *Current Microbiology* **20,** 323-328.

POULAIN, D., HOPWOOD, V. and VERNES, A. (1985). Antigenic variability of *Candida albicans. Critical Reviews in Microbiology* **12,** 223-270.

PUGH, D. and CAWSON, R. A. (1977). The cytochemical localization of phospholipase in *Candida albicans* infecting the chick chorio-allantoic membrane. *Sabouraudia* **15,** 29-35.

QUASH, G., ROCH, A.M., NIVELEAU, A., GRANGE, J. and KEOLOUAN-GRHOT, T. and HUPPERT, J. (1978). The preparation of latex particles with

covalently bound polyamines, IgG and measles agglutinins and their use in visual agglutination tests. *Journal of Immunological Methods* **22,** 165-174.

RAHARY, L., BONALY, R., LEMATRE, J. and POULAIN, D. (1985). Aggregation and disaggregation of *Candida albicans* germ-tubes. *FEMS Microbiology Letters* **30,** 383-387.

RAUVALA, H. and FINE, J. (1979). Structural similarity of the terminal carbohyrate sequence of glycoproteins and glycolipids. *FEBS Letters* **97,** 1-8.

RAY, T. and PAYNE, C. D. (1988). Scanning electron microscopy of epidermal adherence and cavitation in murine candidiasis: a role for *Candida* acid proteinase. *Infection and Immunity* **56,** 1942-1949.

RAY, T. L. , DIGLE, K. B. and PAYNE, C. D. (1984). Adherence of *Candida* species to human epidermal corneocytes and buccal mucosal cells: correlation with cutaneous pathogenicity. *Journal of Investigative Dermatology* **83,** 37-41.

RAY, T. L. and PAYNE, D. (1986). Degradation of basement membrane matrix proteins laminin, fibronectin and type IV collagen by *Candida* proteinase. *The Journal of Investigative Dermatology* **96,** 606.

REGE, V. P. , PAINTER, T. J. , WATKINS, W. M. and MORGAN, W. T. J. (1963). Three new trisaccharides obtained from human group A, B, H and Le^a substance: a possible sugar sequence in carbohydrate chains. *Nature* **200,** 532-534.

RINALDI, M. G. (1993). Biology and pathogenicity of *Candida* species. In *Candidiasis: pathogenesis, diagnosis and treatment*, ed. Bodey, G. P. 2nd ed., pp. 1-20. New York: Raven Press, Ltd.

RIESSELMAN, M.H., KANBE, T. and CUTLER, J. E. (1991). Improvements and important considerations of an ex vivo assay to study *Candida albicans* -splenic tissue interactions. *Journal of Immunological Methods* **145,** 153-160.

ROBERT, R., MAHAZA, C., MAROT-LEBOND, G., TRONCHIN, G. and SENET, J. M. (1991a). Binding of mouse fibrinogen to *Candida albicans in vivo. FEMS Microbiology Letters* **78,** 301-304.

ROBERT, R., JARRIGE, P. L. , MAHAZA, C., COTTIN, J., MAROT-LEBOND, A. and SENET, J.-M. (1991b). Specific binding of neoglycoproteins to *Toxoplasmi gondii* tachyzoites. *Infection and Immunity* **59,** 4670-4673.

ROSEFF, S. A. and SUGAR, A. M. (1993). Oral and esophageal candidiasis. In *Candidiasis: pathogenesis, diagnosis and treatment* ed. Bodey, G. P. 2nd ed., pp. 185-204. New York: Raven Press, Ltd.

ROTROSEN, D., GIBSON, T. R. and EDWARDS, J. E. (1983). Adherence of *Candida* species to intravenous catheters. *Journal of Infectious Diseases* **147,** 594.

ROTROSEN, D., EDWARDS, J. E., GIBSON, J. T. R. , MOORE, J. C., COHEN, A. H. and GREEN, I. (1985). Adherence of *Candida* to cultured vascular endothelial cells: mechanism of attahment and endothelial cell penetration. *Journal of Infectious Diseases* **152,** 1264-1274.

ROTROSEN, D., CALDERONE, R. A. and EDWARDS, J. E. (1986). Adherence of *Candida* species to host tissues and plastic surfaces. *Reviews of Infectious Diseases* **8,** 73-85.

ROY, B. G. and DATTA, A. (1987). A calmodulin inhibitor blocks morphogenesis in *Candida albicans*. *FEMS Microbiology Letters* **41,** 327-329.

ROZALSKA, B., LJUNGH, A., BUROW, A. and RUDNICKA, W. (1995). Biomaterial-associated infection with *Candida albicans* in mice. *Microbiology and Immunology* **39,** 443-450.

RÜCHEL, R., BERNARDIS, F. D., RAY, T. L. , SULLIVAN, P. A. and COLE, G. T. (1992). *Candida* acid proteinases. *Journal of Medical and Veterinary Mycology* **30,** 123-132.

RYLEY, J. F. and RYLEY, N. G. (1990). *Candida albicans* - do mycelia matter? *Journal of Medical and Veterinary Mycology* **28,** 225-239.

SAMARANAYAKE, L. P. and MACFARLANE, T. W. (1980). An *in-vitro* study of adherence of *Candida albicans* to acrylic surfaces. *Archives of Oral Biology* **25,** 603-609.

SAMARANAYAKE, L. P. MCCOURTIE, J. and MACFARLANE, T. W. (1980). Factors affecting the *in vitro* adherence of *Candida albicans* to acrylic surfaces. *Archives of Oral Biology* **25,** 457-465.

SAMARANAYAKE, L. P. and MACFARLANE, T. W. (1981). The adhesion of the yeast *Candida albicans* to epithelial cells of human origin *in vitro*. *Archives of Oral Biology* **26,** 815-820.

SAMARANAYAKE, L. P. and MACFARLANE, T. W. (1982). Factors affecting the *in-vitro* adherence of the fungal oral pathogen *Candida albicans* to epithelial cells of human origin. *Archives of Oral Biology* **27,** 869-873.

SANDIN, R. L. , ROGERS, A. L. , PATTERSON, R. J. and BENEKE, E.S. (1982). Evidence for mannose-mediated adherence of *Candida albicans* to human buccal cells *in vitro*. *Infection and Immunity* **35,** 79-85.

SANDIN, R. L., ROGERS, A.L., BENEKE, E. S. and FERMANDEZ M.I. (1987). Influence of mucosal cell origin on the *in vitro* adherence of *Candida albicans* : are mucosal cells from different sources equivalent?*Mycopathologia* **98,** 111-119.

SANTONI, G., GISMONDI, A., LIU, J. H., PUNTURIERI, A., SANTONI, A., FRATI, L., PICCOLI, M. and DJEU, J. L. (1994). *Candida albicans* expresses a fibronectin receptor antigenically related to α5β1 integrin. *Microbiology* **140,** 2971-2979.

SAWYER, R. T. , GARNER, R. E. and HUDSON, J. A. (1992a). Arg-Gly-Asp (RGD) peptides alter hepatic killing of *Candida albicans* in the isolated perfused mouse liver model. *Infection and Immunity* **60,** 213-218.

SAWYER, R. T., GARNER, R.E. and HUDSON, J. A. (1992b). Effect of lectins on hepatic clearance and killing of *Candida albicans* by the isolated perfused mouse liver. *Infection and Immunity* **60,** 1041-1046.

SCHELD, W. M. , CALDERONE, R. A., ALLIEGRO, G. M. and SANDE, M. A. (1981). Yeast adherence in the pathogenesis of *Candida* endocarditis. *Proceedings of the Society for Experimental Biology and Medicine* **168,** 208-213.

SCHELD, W. M. , CALDERONE, R. A. and BRODEUR, J. P. (1983). Influence of preformed antibody on the pathogenesis of experimental *Candida albicans* endocarditis. *Infection and Immunity* **40,** 950-955.

SCHELD, W. M. , STRUNK, R. W. , BALIAN, G. and CALDERONE, R. A. (1985). Microbial adhesion to fibronectin *in vitro* correlates with production of endocarditis in rabbits. *Proceedings of the Society for Experimental Biology and Medicine* **180,** 474-482.

SCHREVEL, J., PHILIPPE, M., BERNARD, F. and , MONSIGNY, M. (1986). Surface Plasmodium sugar-binding components evidenced by fluorescent neoglycoproteins. *Biology of the Cell* **56,** 49-55.

SEGAL, E., LEHRER, N. and OFEK, I. (1982a). Adherence of *Candida albicans* to human vaginal epithelial cells: inhibition by amino sugars. *Experimental Cell Biology* **50,** 13-17.

SEGAL, E. SKERL, K., CALDERONE, R. and SCHELD, W. M. (1982b). The role of fibronectin in the attachment of *Candida albicans* to human cells. *Clinical Research* 30, 378A.

SEGAL. E,. SOROKA, A. and SCHECHTER, A. (1984). Correlative relationship between adherence of *Candida albicans* to human vaginal epithelial cells *in vitro.* and candidal vaginitis. *Journal of Medical and Veterinary Mycology* **22,** 191-200.

SEGAL, E. and SAVAGE, D. C. (1986). Adhesion of *Candida albicans* to mouse intestinal mucosa *in vitro* : development of the assay and test of inhibitors. *Journal of Medical and Veterinary Mycology* **24,** 477-479.

SEGAL, E., GOTTFRIED, L. and LEHRER, N. (1988). Candidal vaginitis in hormone treated mice: prevention by a chitin extract. *Mycopathologia* **102,** 157-163.

SEGAL, E., KREMER, I. and DAYAN, D. (1992). Inhibition of adherence of *Candida albicans* to acrylic by a chitin derivative. *European Journal of Epidemiology* **8,** 350-355.

SHAPIRO, H. M. (1990). Flow cytometry in laboratory Microbiology: new directions. *American Society for Microbiology News* **56,** 584-588.

SHEPHERD, M. G. , CHIEW, Y. Y. , RAM, S. P. and SULLIVAN., P. A. (1980). Germ-tube induction in *Candida albicans*. *Canadian Journal of Microbiology* **26,** 21-26.

SHEPHERD, M. G. and SULLIVAN, P. A. (1983). *Candida albicans* germ-tube formation with immobilized GlcNAc. *FEMS Microbiology Letters* **17,** 167-170.

SHEPHERD, M. G. (1985). Pathogenicity of morphological and auxotrophic mutants of *Candida albicans* in experimental infections. *Infection and Immunity* **50,** 541-544.

SHEPHERD, M. G. , POULTER, R. T. M. and SULLIVAN, P. A. (1985). *Candida albicans* : biology, genetics, and pathogenicity. *Annual Reviews of Microbiology* **39,** 579-614.

SHEPHERD, M. G. (1987). Cell envelope of *Candida albicans*. *CRC Critical Reviews in Microbiology* **15,** 7-25.

SHEPHERD, M. G. and GOPAL, P. K. (1991). *Candida albicans* : cell wall physiology and metabolism. In *Proceeding of the FEMS Symposium on Candida*

and Candida mycosis ed. Tumbay, E. , Seeliger, H. P. R. and Ang, O. Vol. 50, pp. 21-33. New York: Plenum Press.

SHERWOOD, J., GOW, N. A. R. , GOODAY, G. W. , GREGORY, D. W. and MARSHALL, D. (1992). Contact sensing in *Candida albicans* : a possible aid to epithelial penetration. *Journal of Medical and Veterinary Mycology* **30,** 461-469.

SHIMIZU, K., KONDOH, Y. and TANAKA, K. (1987). Proteinase production and pathogenicity of *Candida albicans*. I. Invasion into chorioallantoic membrane by *C. albicans* strains of different proteinase activity. *Microbiology and Immunology* **31,** 1045-1060.

SHOTTON, D. and WHITE, N. (1989) Confocal scanning microscopy: three-dimensional biological imaging. *Trends in Biochemical Sciences* **14,** 435-439.

SHOTTON, D. M. (1993). Electronic ligth microscopy: the prenciples and practice of video-enhanced contrast, digital intensified fluorescence and confocal scanning light microscopy. In *An introduction to the electronic acquisition of light microscope images* ed. Shotton, D. M. pp. 1-32. New York: Wiley-Liss.

SIMONETTI, N., STRIPPOLI, V. and CASSONE, A. (1974). Yeast-mycelial conversion induced by N-acetyl-D-glucosamine in *Candida albicans. Nature* **250,** 344.

SINOWATZ, F., GABIUS, H. J. and AMSELGRUBER, W. (1988). Surface sugar binding components of bovine spermatozoa as evidenced by fluorescent neoglycoproteins. *Histochemistry* **88,** 395-399.

SKERL, K. G. , CALDERONE, R. A. and SREEVALSAN, T. (1981). Platelet interactions with *Candida albicans. Infection and Immunity* **34,** 938-943.

SKERL, K. G. , CALDERONE, R. A., SEGAL, E., SREEVALSAN, T. and SCHELD, W. M. (1984). *In vitro* binding of *Candida albicans* yeast cells to human fibronectin. *Canadian Journal of Microbiology* **30,** 221-227.

SLUTSKY, B., BUFFO, J. and SOLL, D. R. (1985). High-frequency switching of colony morphology in *Candida albicans*. *Science* **230,** 666-669.

SOBEL, J. D., MYERS, P. G. , KAYE, D. and LEVISON, M. E. (1981). Adherence of *Candida albicans* to human vaginal and buccal epithelial cells. *The Journal of Infectious Diseases* **143,** 76-82.

SOBEL, J. D., MYERS, P. G. , LEVISON, M. E. and KAYE, D. (1982). Comparison of bacterial and fungal adherence to vaginal exfoliated epithelial cells and human vaginal epithelial tissue culture cells. *Infection and Immunity* **35,** 697-701.

SOBEL, J. D., MULLER, G. and BUCKLEY, H. R. (1984). Critical role of germ tube formation in the pathogenesis of *Candida* vaginitis. *Infection and Immunity* **44,** 576-580.

SOLL, D. R. (1988). High-frequency switching in *Candida albicans* and its relations to vaginal candidiasis. *American Journal of Obstetrics and Gynecology* **158,** 997-1001.

SOLL, D. R. (1992). High-frequency switching in *Candida albicans*. *Critical Microbiology Reviews* **5,** 183-203.

SPÖTL, L., MÖST, J. and DIERICH, M. P. (1994). Ca ions stabilize the binding of complement factor iC3b to the pseudohyphal form of *Candida albicans*. *Infection and Immunity* **62,** 1125-1127.

STAIB, F. (1965). Serum-proteins as nitrogen source for yeast-like fungi. *Sabouraudia* **4,** 187-193.

STEWARD, E., HAWSER, S. and GOW, N. A. R. (1989). Changes in internal and external pH accompanying growth of *Candida albicans* : studies of non-dimorphic variants. *Archives of Microbiology* **151,** 149-153.

SULLIVAN, P. A. and SHEPHERD, M. G. (1982). Gratuitous induction by *N*-acetylmannosamine of germ tube formation and enzymes for *N*-acetylglucosamine utilization in *Candida albicans*. *Journal of Bacteriology* **151,** 1118-1122.

SULLIVAN, P. A., YIN, C. Y. , MOLLOY, C., TEMPLETON, M. D. and SHEPHERD, M. G. (1983). An analysis of the metabolism and cell wall composition of *Candida albicans* during germ tube formation. *Journal of General Microbiology* **29,** 1514-1525.

TASCHDJIAN, C. L. and KOZINN, P. J. (1957). Laboratory and clinical studies on candidiasis in the newborn infant. *Journal of Pediatrics* **50,** 426-433.

TASCHDJIAN, C. L. , BURCHALL, J. J. and KOZINN, P. J. (1960). Rapid identification of *Candida albicans* by filamentation on serum and serum substitutes. *American Journal of Diseases of Children* **99,** 212-215.

TIRLAPUR, U. K. and CRESTI, M. (1992). Computer-assisted video image analysis of spatial variations in membrane-associated Ca^{2+} and calmodulin during pollen

hydration, germination and tip growth in *Nicotiana tabacum* L. *Annals of Botany* **69,** 503-508.

THOM, S. M. , BLACKWELL, C. C., MACCALLUM, C. L. , WEIR, D. M. , BRETTLE, R. P. , KINANE, D. F. and WRAY, D. (1989). Non-secretion of blood group antigens and susceptibility to infection by *Candida* species. *FEMS Microbiology Immunology* **47,** 401-406.

THOMAS, D. D., BASEMAN, J. B. and ALDERETE, J. F. (1986) Enhanced levels of attachment of fibronectin-primed *Treponema pallidum* to extracellular matrix. *Infection and Immunity* **52,** 736-741.

TOSH, F. D. and DOUGLAS, L. J. (1991). Effect of blood group and secretor status on the adhesion of *Candida albicans* to mucosal sufaces. In *Proceeding of the FEMS Symposium on Candida and Candida mycosis* ed. Tumbay, E. , Seeliger, H. P. R. and Ang, O. Vol. 50, pp. 127-130. New York: Plenum Press.

TOSH, F. D. and DOUGLAS, L. J. (1992). Characterization of a fucoside-binding adhesin *of Candida albicans. Infection and Immunity* **60,** 4734-4739.

TRONCHIN, G., ROBERT, R., BOUALI, A. and SENET, J.-M. (1987). Immunocytochemical localization of *in vitro* binding of human fibrinogen to *Candida albicans* germ tube and mycelium. *Annales Institut Pasteur Microbiologie* **138,** 177-187.

TRONCHIN, G., BOUCHARA, J. P. , ROBERT, R. and SENET, J. M. (1988). Adherence of *Candida albicans* germ tubes to plastic: ulturastructural and molecular studies of fibrillar adhesins. *Infection and Immunity* **56,** 1987-93.

TRONCHIN, G., BOUCHARA, J. P. and ROBERT, R. (1989). Dynamic changes of the cell wall surface of *Candida albicans* associated with germination and adherence. *European Journal of Cell Biology* **50,** 285-290.

TRONCHIN, G., BOUCHARA, J. P. , ANNAIX, V., ROBERT, R. and SENET, J. M. (1991). Fungal cell adhesion molecules in *Candida albicans. European Journal of Epidemiology* **7,** 23-33.

TSUBOI, R., OGAWA, H., BRAMO, K., RICHARDSON, M. D., SHANKLAND, G.S., CROZIER, W. J. , SEI, Y., NINOMIYA, J., NAKABAYASHI, A., TAKAIUCHI, I., PAYNE, C. D. and RAY, T. L. (1994). Pathogenesis of superficial mycoses. *Journal of Medical and Veterinary Mycology* **32,** 91-104.

TSUCHIYA, T., FUKAZAWA, Y. and KAWAKITA, S. (1965). Significance of serological studies on yeasts. *Mycopathology and Mycology Application* **26,** 1-15.

TYLEWSKA, S. K., FISCHETTI, V. A. and GIBBONS, R. J. (1988). Binding selectivity of *Streptococcus pyogenes* and M-protein to epithelial cells differs from that of lipoteichoic acid. *Current Microbiology* 16, 209-216.

VALENTI, P., VISCA, P., ANTONINI, G. and ORSI, N. (1986). Interaction between lactoferrin and ovotransferrin and *Candida* cells. *FEMS Microbiology Letters* **33,** 271-275.

VAN DE MAYER, L. DESTREE, A.T. and HYNES, R. O. (1983). Fibronectin binds to some bacteria but does not promote their uptake by phagocytic cells. *Science* 220, 201-204.

VANDEN BOSSCHE, H., WILLEMSENS, G., COOLS, W., CORNELISSEN, F., LAUWERS, W. F. and VAN CUTSEM, J. M. (1980). *In vitro* and *in vivo* effects of the antimycotic drug ketoconazole on sterol synthesis. *Antimicrobial Agents and Chemotherapy* **17,** 922-928.

VASILAS, A., MOLINA, L., HOFFMAN, M. and HAIDARIS, C. G. (1992). The influence of morphological variation on *Candida albicans* adhesion to denture acrylic *in vitro*. *Archives of Oral Biology* **37,** 613-622.

VUDHICHAMNONG, K., WALKER, D. M. and RYLEY, H. C. (1982). The effect of secretory immunoglobulin A on the in-vitro adherence of the yeast *Candida albicans* to human oral epithelial cells. *Archives of Microbiology* **27,** 617-621.

WADE, J. C. (1993). Epidemiology of *Candida* infections. In *Candidiasis: pathogenesis, diagnosis and treatment* ed. Bodey, G. P. 2nd ed. pp. 85-108. New York: Raven Press, Ltd.

WALKER, G. M. SULLIVAN, P. A. and SHEPHERD, M. G. (1984). Magnesium and the regulation of germ-tube formation in *Candida albicans*. *Journal of General Microbiology* **130,** 1941-1945.

WANG, J.-R. and STINSON, M. W. (1994). Streptococcal M6 protein binds to fucose-containing glycoproteins on cultured human epithelial cells. *Infection and Immunity* **62,** 1268-1274.

WARDLAW, A. C. (1987). How to deal with proportion data. In *Practical Statistics for Experimental Biologists,* pp. 92-117. Chichester: John Wiley and Sons.

WARREN, N. G. and SHADOMY H.J. (1991) Yeast of medical importance. In *Manual of Clinical Microbiology* ed. Hausler, W. J., Herrmann, K. L. , Isenberg, H. D. and Shadomy, H. J. 5th ed. pp 617-629. Washington D.C.: American Society for Microbiology.

WEBER, W. L. and OSBORN, M. (1969). The reliability of molecular weight determinations by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. *Journal of Biology of Chemistry* **244,** 4406-4412.

WEBSTER, C. E. and ODDS, F. C. (1986). Growth of pathogenic *Candida* isolates anaerobically and under elevated concentrations of CO_2 in air. *Journal of Medical and Veterinary Mycology* **25,** 47-53.

WEINMEISTER, K. D. and DAL NOGARE, A. R. (1994). Buccal cell carbohydrates are altered during critical illness. *American Journal of Respiratory and Critical Care Medicine* **150,** 131-134.

WHELAN, W. L. and MAGEE, P. T. (1981). Natural heterozygosity in *Candida albicans*. *Journal of Bacteriology* **145,** 896-903.

WILKINSON, B. M., MORRIS, L. ADAMS, D. J., EVANS, E.G.V., LACEY, C. J. N. and WALMSLEY, R. M. (1992). A new, sensitive polynucleotide probe for distinguishing *Candida albicans* strains and its use with a computer assisted archiving and pattern comparison system. *Journal of Medical and Veterinary Mycology* **30,** 123-131.

WISNIEWSKI, J. P., MONSIGNY, M. and DELMOTTE, F. M. (1994). Purification of an α-L-fucoside-binding protein from *Rhizobium lupini*. *Biochimie* **76,** 121-128.

WÒJCIAK, B. AND CROSSAN, J. F. (1994). The effect of T cells and their products on *in vitro* healing of epitenon cell microwounds. *Immunology* **83,** 93-98.

YOUNG, G. (1958). The process of invasion and the persistence of *Candida albicans* injected intraperitoneally into mice. *The Journal of Infectious Diseases* **102,** 114-120.

YU, L., LEE, K. K. , ENS, K., DOIG, P. C., CARPENTER, M. R. , STADDON, W., HODGES, R. S., PARANCHYCH, W. and IRVIN, R. T. (1994a). Partial characterization of a *Candida albicans* fimbrial adhesin. *Infection and Immunity* **62,** 2834-2842.

YU, L., LEE, K. K., SHETH, H. B., LANEBELL, P., SRIVASTAVA, G. and HINDSGAUL, O. (1994b) Fimbria-mediated adherence of *Candida albicans* to glycosphingolipid receptors on human buccal epithelial cells. *Infection and Immunity* 62, 2843-2848.