ADHERENCE OF *CANDIDA ALBICANS* TO MUCOSAL SURFACES

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INDEX OF FIGURES

INDEX OF TABLES

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S U M M A R Y

The research described in this thesis was aimed at the characterization of the Candida adhesin, and elucidation of the nature of the human epithelial cell receptor with which it combines. Previous data have shown that the fibrillar mannoprotein layer, produced when Candida albicans is grown in high concentrations of galactose, contains the proteinaceous adhesin. Different Candida species from leukoplakia patients were assayed for their ability to adhere to buccal cells. The results supported previous conclusions that there is a relationship between the ability of different Candida albicans strains to adhere to epithelial cells and their capacity for cell surface modification.

Extracellular polymeric material (EPM) was isolated from culture supernates of C. albicans after growth in medium containing 500 mM galactose. When used to pretreat buccal epithelial cells, EPM inhibited adherence, which suggested that it contains an adhesin that binds to, and blocks epithelial cell receptors.

Fractionation of EPM by affinity chromatography was performed. An index, the adhesion inhibition index (AII) was used to compare the various "lectin-like" components isolated from crude EPM. These studies indicated that use of different buccal cell donors gave different results, with the same C. albicans strain. Attempts to resolve EPM by SDS-polyacrylamide gel electrophonesis had previously proved unsuccessful. However here, success

was achieved using the silver-stain technique.

Chemical and enzymatic digestion of the EPM indicated that the protein portion of the glycoprotein was more important than the carbohydrate at inhibiting adherence. N-Glycanase, papain, mild alkali treatment of EPM, followed by Synsorb-H-2 affinity adsorption chromatography, resulted in a purification of the yeast adhesin of more than 220 fold, relative to the crude EPM (on a protein weight basis).

The nature of the epithelial cell receptor for C. albicans was investigated with potential receptor analogues such as sugars, lectins, monoclonal antibodies and saliva. The adhesion of C. albicans to the buccal cells of blood groups A and O, was found to be significant with respect to secretor status but not blood group. Caution should be shown in the interpretation of sugar inhibition tests. Nevertheless, N-acetyl-D-galactosamine was the most effective single sugar as an inhibitor of adhesion for buccal cell donors of blood group A; N-acetyl-D-galactosamine is the immunodominant blood group sugar for group A cells and the possibility exists that the blood group oligosaccharide on the buccal cell surface functions as the receptor for yeast adhesion.

Further work would be needed to establish whether the purified adhesin had any therapeutic value in treating Candida infections.

ABBREVIATIONS

AII	adherence inhibition index
CMC	chronic mucocutaneous candidosis
ConA	concanavalin A
DEAE	diethylaminoethyl
A_{280}	extinction of a solution in a cell of 1 cm light path at a wavelength of 280 nm
EPM	extracellular polymeric material
M	molar
mA	milli amp
N	normal
NS	not significant
P	probability value
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
SDA	Sabouraud dextrose agar
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
tris	tris (hydroxymethyl) aminoethane
UV	ultraviolet
YNB	yeast nitrogen base

I N T R O D U C T I O N

1 HISTORICAL ASPECTS

'Candida' derives from the Latin name toga candida for the special white robe worn by candidates for the Senate, and albicans is the present participle of the Latin albicare (to whiten). The combination - Candida albicans - thus translates as 'whitening white' (Odds, 1988). The choice between candidosis and candidiasis is one of personal preference. The name 'candidosis' was originally used to describe infections due to Candida albicans, but it has now been shown that other Candida species may be involved, although in fewer cases. Even yeasts of other genera such as Rhodotorula have been described as causes of Candida-like infections, albeit very rarely. Candida infections of virtually every tissue in the human body have been reported, although the most common cases of candidosis are superficial lesions, especially of the mucous surfaces of the mouth or vagina, which are commonly known as 'thrush'. Although there is no certain origin for this name, thrush may have arisen from the Scandinavian equivalent 'torsk' which is used to describe both the disease and the bird (Odds, 1988).

The clinical infection has been recognized since antiquity; Hippocrates described two cases of oral apthae (Adams, 1939), and Samuel Pepys referred to the disease in his diaries on 17th June, 1665 (cited by Winner and Hurley, 1964). In 1839 the fungus was described by Langenbeck; the organism had been seen in buccal apthae

in a case of typhus, but had been incorrectly implicated as the causative agent of typhus. Berg, as cited by Odds (1988), described fully the relationship between the thrush fungus and mouth lesions in 1846. Descriptions of the thrush fungus have all drawn attention to the dimorphic nature of the organism.

2 BIOLOGY OF *CANDIDA*

2.1 Classification

A yeast is defined as a unicellular fungus capable of reproducing by budding or fission (Kreger van Rij, 1984). This is a refinement of Lodder's more general definition (1970) of yeasts as fungi whose predominant morphological form is unicellular.

The genus *Candida* comprises more than 150 species whose main feature is the absence of any sexual form. Yeasts of this genus are asporogenous (Odds, 1988). Generally in microbiology, genera are separated according to their physiological properties and this is so with yeasts. *Candida* species can be identified by assimilation and fermentation tests. Except for the obvious pink colour of the *Rhodotorula* species, colony character is of little value in yeast identification.

The majority of clinical yeast isolates prove to be *C. albicans*. Two rapid and simple tests are available for identification of *C. albicans*: the germ-tube test and

the chlamydospore test. In the germ-tube test C. albicans produces hyphae from blastospores after incubation in serum for three hours at 37°C (Taschdjian et al., 1960). The test for chlamydospore production by C. albicans is generally carried out by growing the yeast on corn meal agar (Benham, 1931) although there are several other recommended media e.g. Czapek-Dox (Dawson, 1962) or maize meal agar (Kligman, 1950). Under phase-constrast microscopy, highly refractile chlamydospores may be observed.

Serological tests have also been used as a means of determining the taxonomy of pathogenic yeasts. Hansenclever and Mitchell (1961) demonstrated the existence of two antigenic types - serotypes A and B. The A serotype possesses the same group of antigens as serotype B, plus two extra antigens. The two types differ in cell wall structure.

Warnock et al. (1979) showed further strain to strain variations. Differing strains of C. albicans exhibited resistance to six different chemicals in agar media.

2.2 Genetics

Odds (1979) reported that "several Candida mutants have been described but genetic studies of yeast pathogens are unknown". Since then progress has been rapid, as reviewed by Shepherd et al. (1985). Indeed,

Odds (1988) devoted a short chapter to this subject.

It has now emerged that natural isolates of C. albicans are diploid. Parasexual fusion, and ultraviolet irradiation inducing mitotic crossing over have been used to analyse the genetic similarity of strains (reviewed by Shepherd et al., 1985). Poulter et al. (1981) first suggested that C. albicans was naturally diploid, and Sarachek and Weber (1984) confirmed this by means of parasexual fusion. Whelan (1987) described in detail the genetics of C. albicans, C. glabrata and C. tropicalis. In that review he noted that in an asexual, diploid species such as C. albicans, products of mutagenesis may arise by de novo mutation or by mitotic recombination and segregation, and he therefore proposed the term 'hereditary variant' as preferable to mutant in describing strains with abnormal properties to avoid implying a process of de novo mutation simultaneously in two genes.

Variants of C. albicans have been isolated, complemented with each other, and recombination has been performed. Using parasexual genetic procedures, five linkage groups have been established (Shepherd et al., 1985). C. albicans frequently gives rise to variant colony forms. In 1985, two laboratories described colony variants. Pomés et al. (1985) showed rough and smooth sectors of a mutant of C. albicans that had been exposed to ultraviolet light, and Slutsky et al. (1985)

characterised seven interconvertible forms of colony growth, with high frequency switching taking place between them. A second phenotypic switching system was described by Slutsky et al. (1987) between "white" and "opaque" colonies.

2.3 Ecological niche

Yeasts are widely distributed in animal, plant and terrestial aquatic environments (Do Carmo-Sousa, 1969). Yeasts which are the causal agents of human candidosis have a wide natural distribution in primates, farm and domestic animals, rodents, birds and marsupials. C. albicans is by far the most pathogenic, as well as the most common species of Candida occurring in humans. Candida species are frequently found in the hospital environment - in foods, in the air, on the floor, and on other surfaces in hospital wards (Barnett et al., 1983). Although the yeasts causing candidosis are found throughout the animal kingdom, the most important source of Candida in human disease is the human reservoir i.e. gut, mouth, and vagina. There are enormous disparities in the frequencies of isolation of yeasts from humans. Gentles and La Touche (1969) concluded that C. albicans and a few other yeast species were harmless commensals of the mucous membranes and digestive tracts of normal individuals.

2.4 Morphogenesis

2.4.1 Morphological variability

Depending on the growth conditions, C. albicans takes on different morphological forms, from a true mycelium to budding yeasts. The blastospore is the unicellular form of the fungus - distinguished by a specific mode of cell division known as budding. The hyphae are long microscopic tubes comprising multiple fungal cells, separated by septa. The study of morphogenesis in dimorphic fungi is crucial in the diagnosis and treatment of fungal infections, and in pathogenicity.

2.4.2 Dimorphism and pathogenicity

There are many accounts of experimental studies with C. albicans that report an association between mycelium formation and infection (Balish and Philips, 1966; Gresham and Whittle, 1961; Louria and Brayton, 1964). These reports are based on the fact that the mycelial form is invariably seen in smears or scrapings within hours of inoculation of C. albicans into experimental animals.

Rapid morphological conversion from budding yeasts to the mycelial form in tissues suggests pathogenicity of this form. Nevertheless, controversy surrounds the invasive potential of the two forms; for example, Russell and Jones (1973) identified no difference between them. When working with rabbits and mice, Evans and Mardon

(1977) found yeast cells to be more virulent than pseudohyphal cells; and further that yeast cells were more virulent than hyphal forms because of less effective host defence mechanisms against the yeast phase of C. albicans (Evans, 1980). These differences were in part attributed to differences in the structural integrity of the cell wall influencing host defence mechanisms, which were more effective against the hyphal than the blastospore phase. Savage and Balish (1971) found that mycelium deficient variants of C. albicans occurred spontaneously in vitro or could be isolated from cases of candidosis, thus bringing into question the validity of the requirement for the yeast-mycelium conversion. Martin et al. (1984) concluded that the pathogenic potential of C. albicans appears to depend on germ-tube formation.

The change from yeast to hyphal form is environmentally regulated (Soll, 1986). The molecular determinants remain unclear. Production of filaments may be a process associated with the adhesion of the invading C. albicans, and penetration of the host cell. Conditions such as temperature, pH, and the microenvironment of the host may favour hyphal development in vitro. Sobel et al. (1984) found that a variant strain of C. albicans, incapable of hyphal formation at 37°C, was less likely to colonize the rat vagina than the wild-type strain. Thus hyphal production

by C. albicans may be considered an important but not essential virulence factor.

3 CELL SURFACE OF CANDIDA ALBICANS

3.1 Cell wall composition

The cell wall of the baker's yeast Saccharomyces cerevisiae has been the subject for most of the studies on the structure of yeast cell walls. The cell wall of C. albicans has been investigated less intensively. It is multilayered in structure (Scherwitz, 1982) and composed of glucans (β1,3 and β1,6 polymers of glucose), mannoprotein, chitin (a β1,4 polymer of N-acetyl-D-glucosamine), protein and lipid (Phaff, 1971). The chitin is thought to be located in the bud scar, although glucan and mannoprotein are present all over the wall.

Evidence suggesting the extracellular location of mannose polymers has come from studies with anti-mannan antibodies (Ballou, 1976) and concanavalin A (Tkacz and Lampen, 1972). Chattaway et al. (1968) identified differences in the quantities of chitin in the cell walls of blastospores and hyphae in C. albicans. The β1,3 linked glucan appears to structure the cell shape. Purified β1,3 glucanase can almost completely solubilize isolated cell walls (Zlotnik et al., 1984). These latter studies indicated that mannoproteins shield the cell wall

glucan from attack by glucanase. Although removal of mannoproteins does not cause cell death, the cell remains vulnerable to any basic or positively-charged electrolyte in the surroundings. Mannoprotein extraction does not destroy cell wall shape and led Zlotnik et al. (1984) to infer that mannoprotein is packed on to the glucan layer; it is a filling material enmeshed in the glucan structural network. Any loosening of this mannoprotein layer results in increased porosity of the Candida cell wall.

3.2 Chitin

Chitin is a β1,4 polymer of N-acetyl-D-glucosamine. Chattaway et al. (1968) found that the amount of chitin in the mycelial form of C. albicans was about three times higher than in the budding form of the organism. Chitin is less evident in the cell walls of yeast than in the cell walls of many filamentous fungi, and was first identified in Sacch. cerevisiae cell walls by its characteristic X-ray diffraction pattern which was similar to that of crustacean chitin (Phaff, 1971). The physical nature of chitin in the wall is only partially understood.

3.3 Mannan/Mannoprotein

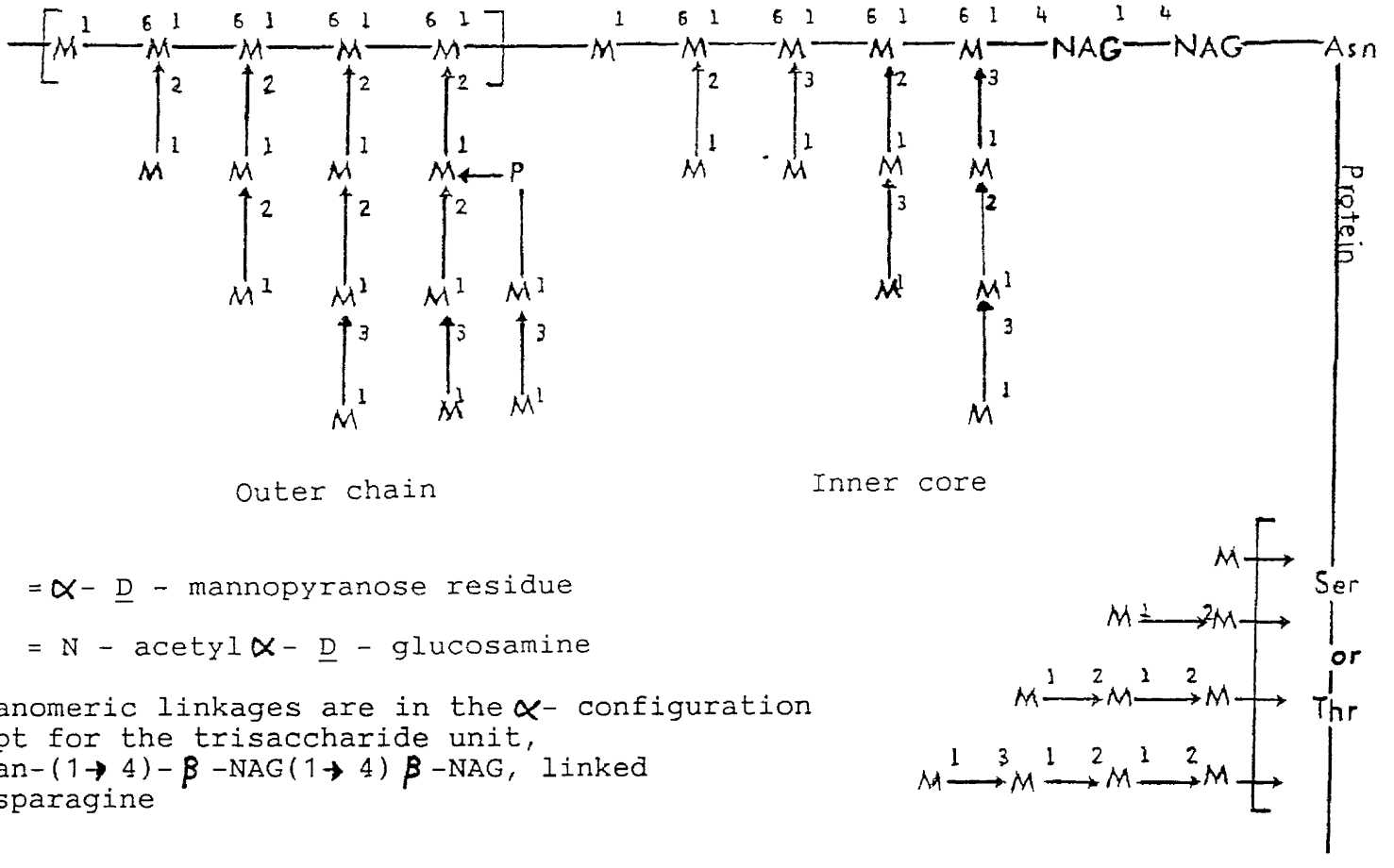
Yeast mannan was discovered in 1894 by Salkowski and called 'yeast gum' (Phaff, 1971). Chemical

investigations of yeast mannan have been carried out on preparations extracted from intact cells with dilute alkali (2% KOH, 100°C) and then precipitated as an insoluble copper complex (Arnold, 1983). Peat et al. (1961) examined mannan extracted by autoclaving intact yeasts at neutral pH. Mannan consists of long chains of α1,6 linked mannose residues with shorter side chains containing α1,2 and α1,3 linkages.

The protein-mannan link is identical to that in many other glycoproteins (Nakajima and Ballou, 1974). Immunological studies indicate that in C. albicans, mannoprotein is located on the cell surface (Hasenclever and Mitchell, 1964). The general features of its structure are similar to those of Sacch. cerevisiae cell wall mannoprotein (Ballou, 1976).

Mannoproteins are easily attacked by dilute alkali, dithiothreitol and pronase, which probably degrade/hydrolyse the protein that holds the polymannose chains together. Analysis of these degradation products renders a generalized structure for mannoprotein (see Figure 1). Two kinds of linkage between carbohydrate and protein parts of Sacch. cerevisiae were described by Nakajimi and Ballou (1974) and Ballou (1976). Alkali treatment cleaves small oligosaccharides from crude mannoprotein preparations (β-elimination) releasing fragments linked to serine and threonine in the protein. Most of the carbohydrate is attached to asparagine

Figure 1 Structure of mannan from Saccharomyces cerevisiae X 2180



M = α- D - mannopyranose residue

NAG = N - acetyl α- D - glucosamine

All anomeric linkages are in the α- configuration except for the trisaccharide unit, β -Man-(1→ 4)- β -NAG(1→ 4) β -NAG, linked to asparagine

Base labile oligosaccharides

residues in the protein by di-N-acetylchitobiose links. Nakajima and Ballou (1974) confirmed this linkage between mannoprotein to be identical with that found in many glycoproteins. A second moiety of mannan consisting of α1,2 and α1,3 linked oligosaccharides attach at their reducing ends by an O-glycosidic link to serine or threonine. The two C. albicans serotypes A and B (Hasenclever and Mitchell, 1964) can be differentiated by the extent of branching, length of side chain, and the proportion and position of the α1,3 bonds in the side chain (Phaff, 1971). Diedrich et al. (1984) used papain to release mannopeptides from C. albicans cell walls and the material extracted in this way co-migrated on dextran gels with mannopeptides from culture filtrates. They concluded that these mannopeptides were remnants of proteolytic turnover in cell wall polysaccharides.

Less well researched than the carbohydrates of the cell wall in C. albicans are the levels of phosphorus. Higher levels of phosphorus are reported in C. albicans mannan than in Sacch. cerevisiae. Stewart and Ballou (1968) reported a mannose:phosphorus ratio of 18:1 in C. albicans compared to one of 144:1 in Sacch. cerevisiae.

3.4 Glucans

'Glucan' refers to a large group of D-glucose polymers that vary in the type and proportion of individual glycosidic bonds. Glucans in the yeast cell

wall are generally β D-glucans with β1,3 linkages which may occasionally be interrupted by β1,6 sequences (Peat et al., 1961). Bacon and Farmer (1968) showed by methylation analysis and periodate oxidation of the alkali-extracted glucan, from C. albicans, that it is highly branched; 73% are β1,6 linkages, the rest being β1,3 bonds. It is possible that proportions of β1,3 and β1,6 links may differ in cell walls from yeast and hyphal forms (Gopal et al., 1984). The physiochemical properties of β-glucan, its insolubility and high degree of crystallinity, indicate that its synthesis probably occurs in situ i.e. in the cell wall or at the outer surface of the plasmalemma (Sentendreu et al., 1975).

Andaluz et al. (1986) suggested that the glucan molecule is synthesized while bound to an acceptor other than glucan, which is subsequently excised, probably during the transfer of the glucan moiety to the cell wall glucan.

3.5 Plasma membrane

This important organelle has received limited attention at the biochemical level. However, plasma membrane-enriched fractions have been characterized from the yeast and mycelial forms of C. albicans. Analysis indicated that the major components were protein and lipid (Marriot, 1975, a and b). Membranes from the yeast form contained 50% protein, 45% lipid, 9% carbohydrate

and 0.3% nucleic acid. Significant differences in the levels of phospholipid free esterified sterols and total fatty acids of membranes, were seen between the two morphological forms of the organism (Marriot, 1975a). Enzyme activity in the plasma membrane of the two forms of the organism has also been given little detailed attention (Marriot, 1975b). However, mannan synthase may reside here along with a magnesium-dependent ATP-ase. This is likely to be involved in the establishment of proton gradients for solute uptake (Blasco and Gidrol, 1982).

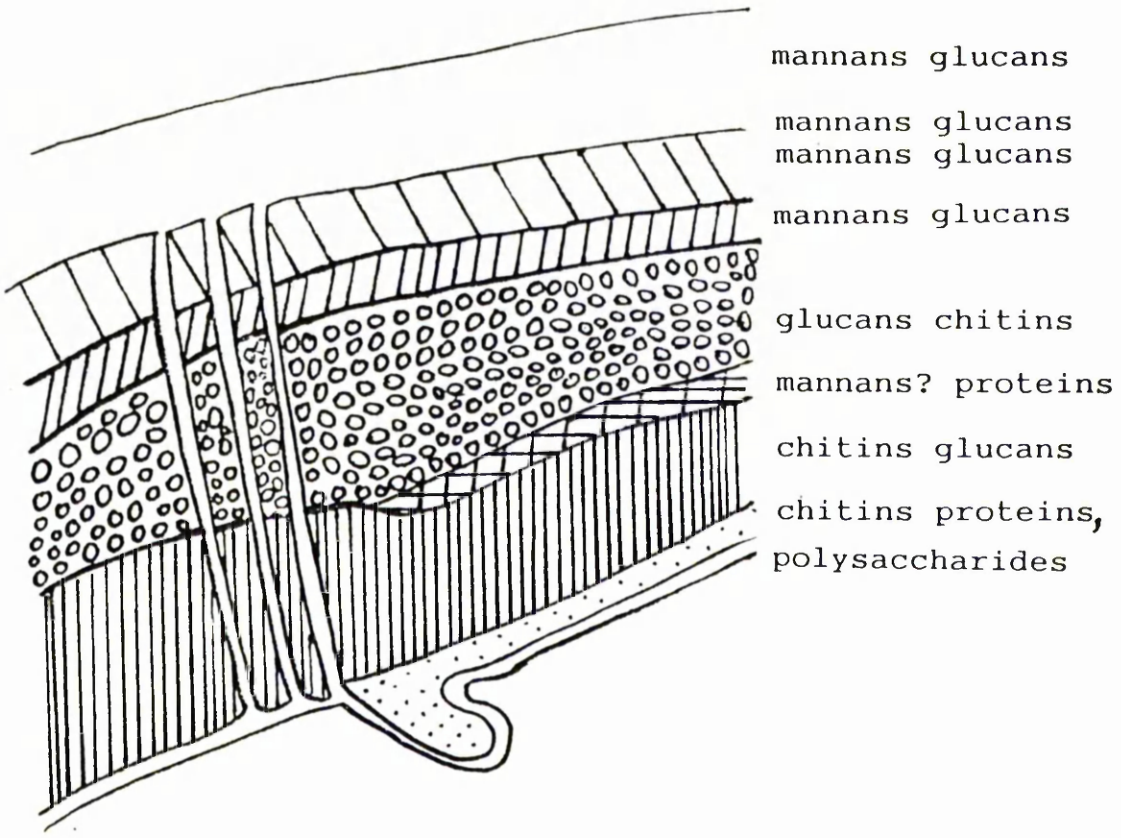
3.6 Cell wall ultrastructure

The cell wall of C. albicans is important for several reasons. Firstly, the cell surface appears to mediate the adhesion of parasite to host. Secondly, the cell surface plays a role in determining the cell's immunity to certain drugs such as polyene antibiotics (Gale et al., 1975), and finally it contains antigenic structures which are involved in serotyping (Yu et al., 1967).

The structure of the cell wall of C. albicans appears complex compared to that of Sacch. cerevisiae and by comparison our knowledge is limited. Work on the ultrastructure of the wall of C. albicans has revealed that it is composed of four (Yamaguichi, 1974), five (Djaczenko and Cassone, 1972) or eight (Poulain et al., 1978) distinct layers.

According to Cassone et al. (1973) the cell wall of C. albicans is organized into a multilayered structure with amorphous, granular and fibrous components, with varying electron densities. Tronchin et al. (1984) utilized a periodic acid thiocarbohydrazide-silver proteinate technique (PATAg) of staining in conjunction with electron microscopy to reveal a fibrillar layer of filaments arranged at right angles to the cell surface. Djaczenko and Cassone (1972) used transmission electron microscopy to visualize five distinct layers and a fibrillar floccular layer on the yeast cell surface. Takamiya et al. (1984) employed post-embedding immunoferritin tests with ultra-thin sections of C. albicans to demonstrate mannan antigens on the exterior of the cell surface, as well as in the cytoplasm near the cytoplasmic membrane, thus indicating that mannan antigens are transported from the cytoplasm via channel-like organelles in the cell wall to their final location in the exterior cell wall layer (see Figure 2).

The ultrastructural features of the host parasite relationship in oral candidosis had not been described until Montes and Wilborn (1968) showed an electron dense material present on the external wall of the cell hyphae which had penetrated host epithelial cells. This extracellular material was much condensed along a certain length of the hyphal tips (Rajasingham and Cawson, 1982). Marrie and Costerton (1981) demonstrated that a ruthenium



Cell wall structure of C. albicans with channels for mannoprotein transportation

Based on Poulain et al. (1978)

and modified by Takamiya et al. (1984)

red positive matrix mediates adhesion of C. albicans blastospores to epithelial cells and this finding accorded with Mohamed (1975) where the fungus was seen to have a thick cell wall, surrounded by an outer floccular layer.

3.7 Extracellular polymeric material (EPM)

C. albicans excretes polysaccharide-protein complexes into the nutrient medium (Masler et al., 1966) consisting of 74-86% mannan, 21-31% glucose, 1-1.5% glucosamine and 11-14% protein; this material was immunologically active. Diedrich et al. (1984) characterized a low molecular weight extracellular material produced by metabolically active C. albicans. They showed that three strains of C. albicans produced a low molecular weight extracellular substance that could be fractionated into two components by ion-exchange chromatography, and further that these components were the remains of proteolytic turnover of cell wall glycoproteins. Papain treatment of the cells also generated this material.

Biological activities have been seen with extracellular polymeric material from C. albicans. Mankowski (1968) reported that this material inhibited the growth of newborn mice and also had cidal effects on these mice.

Masler et al. (1966) isolated EPM with a molecular weight of about 200,000 from glucose-containing nutrient

media after cultivation of slightly, moderately and highly virulent strains of C. albicans. All the complexes were immunologically active against specific antisera prepared against moderately virulent strains.

Šikl et al. (1969) found that glycoprotein from C. albicans released histamine from most cells and Trnovec et al.(1978) reported that this material was able to depress the phagocytic ability of white cells. Saltarelli and Coppola (1980) used an extracellular polymeric substance from C. albicans to treat Sarcoma 180 tumour cells in Swiss female mice. Extracellular polymeric material from C. albicans inhibited attachment of yeasts to neutrophils (Diamond et al., 1980) and adherence to buccal cells (McCourtie and Douglas, 1985). Extracellular glycoproteins were part of the outer fibrillar layer which was readily released from the cell surface (McCourtie and Douglas, 1981). This material is presumed to contain the adhesins capable of interacting with epithelial cell surface receptors. Extracellular polymeric material isolated by prolonged dialysis of culture supernatants (Critchley and Douglas, 1987a) was similar to that obtained previously (McCourtie and Douglas, 1985), comprising about 70% carbohydrate, 10% protein and 0.5% phosphorus.

4 CANDIDOSIS

Candida infections have been reported in virtually every tissue of the human body; however the different forms fall into two general categories - superficial and systemic. Superficial is intended to mean 'on the surface' e.g. of the oral mucosa and on the skin and nails. Candida infections of organs e.g. kidneys, lungs etc. may be considered deep-seated or systemic.

4.1 Factors influencing Candida infections

Since yeast infection, as opposed to yeast carriage is a comparatively rare event it follows that in normal circumstances an equilibrium between the host and the yeast microflora ensures the avirulent, commensal status of the latter (Odds, 1988). Gentles and La Touche (1969) listed 39 disorders, diseases, disabilities and iatrogenic factors that predispose individuals to candidosis.

4.1.1 Dietary factors

Gentles and La Touche (1969) listed carbohydrate rich diet as a factor predisposing to candidosis. Samaranyake and MacFarlane (1985) presented their hypothesis, describing how high concentrations of carbohydrate might aggravate oral candidosis. The effect of iron deficiency on oral candidal infections is difficult to ascertain, yet studies indicate that iron deficiency in concert with

a variety of other dietary factors e.g. vitamin deficiency, may be involved in the pathogenesis of oral candidosis (Jorrizo, 1982; Samaranayake, 1986).

4.1.2 Mechanical factors

Trauma, local occlusions and maceration of tissues predispose the host to candidosis. Local maceration of the skin occurs in many common situations. Workers who frequently immerse their hands in water are particularly prone to candidosis of the nails, nailfolds and finger clefts. These forms of candidosis are the only real instances of Candida as an agent of occupational disease. The same problem is encountered in children who suck their thumbs (Odds, 1979). C. albicans is found rarely on the skin; however, the probability of its recovery from moist areas e.g. groin and toewebs is far higher than from the general skin surface (Somerville, 1972). Maceration of the skin or membranes so as to give rise to increased local humidity may predispose to yeast overgrowth and infection (Odds, 1988).

4.1.3 Medical factors

Medical factors increasing host susceptibility to Candida albicans include some drugs and various surgical techniques. Patients receiving broad spectrum antibiotics, or multiple narrow-spectrum antibiotics or corticosteriods appear to be more susceptible to yeast overgrowth and consequent yeast infection (Odds, 1988).

These drugs suppress the endogenous microflora or suppress the host defence mechanisms against infections. Liljemark and Gibbons (1973) investigated the effect of antibiotics on gnotobiotic mice and showed that C. albicans colonization was inhibited by Streptococcus salivarius and Streptococcus miteor. Attachment inhibition by indigenous flora may partly explain the suppression of Candida colonization.

Immunosuppressive drugs may also create an environment favourable for host invasion by C. albicans as found after bone marrow transplant (Bandini et al., 1986). In experimental animals, cyclophosphamide selectively depletes the immune system - reducing the peripheral blood polymorphonuclear leucocytes as well as spleen cellularity, creating increased host susceptibility to C. albicans infection (Bistoni et al., 1984).

Oral contraceptives may activate vaginal candidosis. Lopez-Martinez et al. (1984) found that the most frequent predisposing factors were in decreasing order of frequency - pregnancy, the association of pregnancy and malnutrition, anaemia and malnutrition. Evidence suggesting that the oestrogen contraceptive 'pill' predisposes to candidosis has become a matter of subsequent controversy. Odds (1979) concluded that oral contraceptives containing oestrogen enhance the susceptibility of the vagina to yeast overgrowth, which

in turn leads to symptons of yeast vaginitis.

Of the therapeutic procedures likely to potentiate infection, indwelling catheters and intravenous feeding are among the most important. Recently an outbreak of Candida parapsilosis septicaemia was reported and this was associated with contaminated intravenous feeds (Solomon et al., 1984). Marrie and Costerton (1984a) used scanning electron microscopy and transmission electron microscopy to view in situ bacterial colonization of catheters. Surfaces of catheters are generally imperfect; infusites may create a biofilm. Marrie and Costerton (1984b) also indicated that bacteria and yeasts colonize catheters. These catheters were removed from a patient with C. albicans peritonitis and yeast cells were found to be embedded in the amorphous background.

4.1.4 Natural factors

Natural factors predisposing the host to candidosis include other microbial infections, endocrine dysfunctions and defects in cell mediated immunity (Odds, 1988). Candida vulvovaginitis has been described as a common presenting sympton of diabetes mellitus (Nagesha and Anathakrishana, 1970).

Previous investigators have reported higher than normal frequencies of carriage of yeasts from the vagina, urine and oropharynx of diabetics (Barlow and Chattaway, 1969). High blood and tissue glucose levels or low skin lactate levels may favour the growth of Candida in

diabetics. Diabetic women have high glycogen levels so that the effect of diabetes on the vagina resembles that of pregnancy. There appear to be no differences in *in vitro* phagocytosis of *C. albicans* between diabetic and normal leucocytes (Davies and Denning, 1972).

Segal *et al.* (1984) found that vaginal epithelial cells from fecund women who were pregnant or diabetic, had a greater propensity to bind *C. albicans* than did vaginal epithelial cells from non-diabetic controls. Maternal vaginal candidosis appears to be the major source of neonatal oral thrush (Davidson *et al.*, 1984). During passage of the foetus through the colonized vagina, the infant may ingest some of the vagina contents (Jennison, 1977). Other factors predisposing to the development of oral thrush include prematurity, low birth weight, previous antimicrobial therapy, prolonged labour with resucitative procedures and contact with nipples and bottles contaminated with fungi (Jennison, 1977).

Other factors may also be important in the development of oral thrush since only 10-24% of infants harbouring *C. albicans* in their oral cavity develop thrush (Lay and Russell, 1977). During the first week of life there is an increase in the adherent capacity of buccal epithelial cells to *C. albicans*. It is postulated that this increase may have a role in the development of oral thrush.

4.2 Oral candidosis

Epstein et al. (1984) described several categories of oral candidosis. Diagnosis depends on the presence of the organism in a direct smear, the culture of significant numbers of the organism and, indirectly, upon the efficacy of the antifungal agents that are prescribed.

4.2.1 Acute pseudomembraneous candidosis (oral thrush)

In this, the classically recognized form of candidosis, C. albicans invades the epithelial layers of the oral mucosa but never appears to penetrate deeper than the stratum corneum (Lehner, 1967). The infection is usually painless, and is characterized by white patches (apthae) that appear as discrete lesions on the surface of the buccal mucosa, throat, tongue and gum linings. Coalescence results in pseudomembranes. Lesions may be rubbed off leaving red raw epithelium. The disease is most prevalent in the very young, the old and the terminally ill (Finlay, 1986). A prevalence of 15% was recorded in debilitated elderly patients: therefore the incidence among the elderly population as a whole is likely to be lower.

4.2.2 Chronic atrophic candidosis - denture stomatitis

Chronic atrophic candidosis was not mentioned by Winner and Hurley (1964) and knowledge of its association with Candida arises from more recent studies. Both

Cawson (1966) and Budtz-Jörgensen (1971) firmly established the link between Candida and denture-related palatal inflamation.

Denture stomatitis is characterized by chronic erythema and oedema of that portion of the upper palate which comes into contact with the dentures. Although patients may experience slight soreness, the presenting complaint is angular cheilitis.

Cawson (1966) and Davenport (1970) found that denture stomatitis was four times as prevalent among female as male denture wearers. Repeated oral sucrose rinses aggravate existing lesions in patients with denture stomatitis, and initiate infection in lesion-free denture wearers (Olsen and Birkeland, 1976). Denture liners and adhesives do not inhibit the growth of C. albicans (Williamson, 1968), although antifungal agents may be incorporated into denture liners as an alternative treatment (Douglas and Walker, 1973).

4.2.3 Chronic hyperplastic candidosis - candidal leukoplakia

Chronic hyperplastic candidosis exhibits chronic, discrete, localized white lesions of variable size that are hard and rough to touch (Odds, 1988). As in the case of oral thrush, Candida tissue invasion in leukoplakia is restricted to superficial epithelial layers (Mohamed, 1975). Krogh et al. (1986) suggested that there may be phenotypic differences between C. albicans strains

associated with leukoplakias and normal epithelia in the same patient.

4.3 Vaginal candidosis (vaginal thrush)

Hurley (1975) recognised vaginal thrush as the most prevalent disease of pregnancy, and recorded an incidence of 15% - 16%. With this form of candidosis, sexual intercourse often becomes uncomfortable and painful (Witkin et al., 1986).

Kinsman and Collard (1986) indicated that in rat vaginas, predisposing factors to C. albicans infections included a cornified epithelium and the absence of leukocytes - these conditions were present in oestrous. Edman et al. (1986) reported that mild zinc deficiency may play a role in susceptibility to recurrent vaginal infections. Throughout pregnancy, the main predisposing factor may be an indirect effect on the immune system due to hormonal changes (Mathur et al., 1978).

A further predisposing factor may be the long-term and unjudicious use of broad spectrum antibiotics which have a detrimental effect on the normal bacterial flora. In the absence of these bacteria, yeast growth and fungal pathogenicity is stimulated (Knight and Fletcher, 1971). Since systemic fungal disease frequently occurs because of a breakdown of the host's immune system (Stenderup and Schønheyder, 1984), it is possible that vaginal candidosis could have a similar predisposing cause

(Ryley, 1986). However a defective cell mediated immune response may not, by itself, always lead to recurrent candidal infection (Witkin et al., 1986).

During the sexually active years, the healthy vagina is populated by a variety of bacteria, including lactobacilli. These produce lactic acid which is largely responsible for the acidic nature (pH 4-5) of the vagina.

However there is little correlation between pH and vaginal candidosis (Ryley, 1986). In order to eliminate potential reinfection therefore, the source of C. albicans must be eliminated. Although in the majority of cases, topical treatment with various azole preparations or oral treatment with ketoconazole will provide quick symptomatic relief, such treatment does nothing to correct the initial predisposing factor (Ryley, 1986) and oral treatment is preferred.

4.4 Chronic mucocutaneous candidosis

Chronic mucocutaneous candidosis (CMC) is an uncommon superficial candidal infection of the skin, nails, and oral genital mucosa (Jorrizo, 1982). It is reputedly not a single disease entity, but rather a final common pathway for multiple predisposing abnormalities of the immune system.

CMC often affects children during their first decade of life, and, in many cases, before they are two years

old. Mature onset cases are rarely seen (Odds, 1988). Patients often show several demonstrable immune defects (Lehner et al., 1972).

The first Candida lesion to appear in cases of CMC is the typical form of oral thrush. Angular cheilitis and lip fissures may develop, and infection may spread to the larynx and, rarely, to the oesophagus. Odds (1979) tabulated nine principal diseases and disorders associated with CMC. Patients with CMC are generally refractive to topical antifungals (Jorrizo, 1982). Ketoconazole has been successfully used to treat CMC with minimal side-effects (Graybill et al., 1980).

4.5 Systemic candidosis

In contrast to the common Candida infections of mucous membranes and skin, candidosis infections of the deep organs are rarely seen. Such infections present in different ways depending on the site of invasion, the effectiveness of the host's immune response and the presence of underlying disease (Myerowitz et al., 1977).

Systemic candidosis is difficult to diagnose clinically because there is no typical clinical picture (De Repentigay and Reiss, 1984). However Burnie and Williams (1985) evaluated a commercial latex agglutination test, which reliably differentiated between colonization of intravenous catheters and systemic infection. Although the test failed to detect some cases

of systemic infection, at a titre of greater than 1:8 it is virtually diagnostic of systemic disease and is far easier to perform than the radioimmunoassay (Weiner and Coates-Stephen, 1979).

Although rare, nosocomal candidosis has recently been documented (Isenberg et al., 1989). An environmental reservoir for those Candida species involved in nosocomal infections has not yet been established (Burnie et al., 1985). In the London outbreak of systemic C. albicans (Burnie et al., 1985), the strain not only affected patients in the intensive care unit, but was also isolated from oral swabs taken from four nurses working in the unit, and from the hands of one of the nurses. No environmental source could be identified. The strain exhibited enhanced survival in handwashing experiments and was relatively resistant to 'Hibiscrub' (chlorohexidine). Oral ketoconazole reduced the rate of isolation of the outbreak strain from patients. Collingnon and Sorrell (1983) described systemic candidosis in heroin addicts and Hay (1986) provided a short review on this. Isenberg et al. (1989) found that a single nurse infected eight patients and that following her removal from nursing responsibilities, the cluster outbreak ended.

4.5.1 Extrahepatic bilary tract candidosis

Although candidosis has been reported in virtually every organ, involvement in the extra hepatic bilary

tract is rare (Irani and Truong, 1986). Clinical and laboratory findings are not distinctive enough to suggest a specific diagnosis, but right hand upper quadrant pain coupled to jaundice could be considered markers. If suitable, surgery to remove a fungal ball blocking the bile duct, along with antifungal drugs generally provides for recovery.

4.5.2 Renal candidosis

In both humans (Barnes et al., 1983) and animals (Hasenclever and Mitchell, 1962), the organ most susceptible to infection appears to be the kidney. Renal candidosis has been diagnosed predominantly in middle aged and elderly patients. Its clinical and diagnostic features are fever and urinary obstruction.

The mechanism by which C. albicans, once localized, invades renal parenchyma is as yet undefined (Barnes et al., 1983). Lee and King (1983) stated that blood-borne C. albicans cells are rapidly cleared from the circulation by the liver, lungs, spleen and kidney. Barnes et al. (1983) suggested that the localization of C. albicans blastospores within renal vasculature occurred primarily by adherence to endothelial surfaces. This was substantiated by electron microscopy showing numerous fibrils extending from the yeast surface to the endothelium.

4.5.3 Gastrointestinal candidosis

De Maria et al. (1976) suggested that the development of systemic candidosis involves gastrointestinal colonization followed by mucosal invasion and dissemination via the bloodstream. Symptoms include loss of appetite, nausea, vomiting and intestinal pain (Odds, 1988).

C. albicans has been shown to be able to cross the human bowel wall (Pope and Cole, 1981) and therefore entry of C. albicans into the body via the gastrointestinal tract (GI tract) is important. Indeed, Stone et al. (1974) suggested that this may be the primary invasion mechanism. Krause et al. (1969) previously investigated this theory. Two hours after ingestion of C. albicans in saline suspension, typical symptoms of septicaemia developed - chills, headache and high fever. Nystatin administration via oral and catheteric routes gave almost immediate remission.

Auguste and Nava (1986) recognised the difficulty in diagnosis of this form of infection. A trial of clotrimazole has confirmed its efficacy in gastrointestinal forms of candidosis and the compound has been claimed to prevent the extension of oral thrush into the oesophagus (Shechtman et al., 1984)

4.5.4 *Candida* endocarditis

The signs of endocarditis are generally fever, chills, anorexia, nausea, vomiting and heart failure (Odds, 1988). These symptoms and the condition were rarely seen before the introduction of antibiotics, steroids and cardiac surgery (Chaudhuri, 1970). The increased frequency of the disease usually appears in three clinical settings - prosthetic valvular insertion, intravenous drug abusers, and as a complication of prolonged intravenous antibiotic administration, or hyperalimentation (Scheld *et al.*, 1983).

Although Marrie *et al.* (1984) used scanning electron microscopy and transmission electron microscopy to describe the ultrastructure of *C. parapsilosis* endocarditis, there is no reliable clinical or laboratory indicator other than surgically invasive exploration for a definitive diagnosis (Odds, 1988). Rapid diagnosis is however, the most important factor in the successful management of *Candida* endocarditis. Seelig *et al.* (1979) proposed vigilance, and prophylactic monitoring of patients at risk from the disease should help reduce the mortality rate. Rose (1978) recommended that following the removal of intravenous catheters, repeated blood-cultures may lead to an earlier diagnosis of *Candida* endocarditis.

4.5.5 *Candida* endophthalmitis

Graham *et al*. (1986) presented four cases of *Candida* endophthalmitis to demonstrate the difficulties of both ocular and microbiological diagnosis. The initial complaint is of painful photophobia and of particles floating in the field of vision. The disease is always associated with *Candida* septicaemia. Once endophthalmitis has been diagnosed, antifungal drugs should be given although their efficiency is not absolute. The preferred regime is probably fluconazole and ketonazole (Graham *et al*., 1986).

5 ANTIFUNGAL AGENTS

Numerous antifungal agents are active against *Candida* species. The more recent ones can be divided into three classes; the polyenes, 5-fluorocytosine and the ergosterol biosynthesis inhibitors. These antifungal drugs, and their effects have been reviewed by Vanden Bossche *et al*. (1987).

5.1 The polyene macrolide antibiotics: amphotericin B and nystatin

Nystatin and amphotericin B are characterized by a ring of carbon atoms containing both a system of conjugated double bonds and a hydrophilic region characterized by the number of C atoms in the ring (Kerridge and Nicholas, 1986).

Nystatin was the first polyene ever to be applied to the treatment of candidosis and was first isolated from Streptococcus noursei or Strep. albus (Hamilton-Miller, 1973). It is primarily used to treat Candida infections of the skin, intestinal tract and mucous membrane. Vaginal candidosis usually responds well to nystatin.

At the minimum growth inhibitory concentration, the nystatin interacts with sterol-containing plasma membranes rendering them permeable (Palacios and Serrano, 1978). This dissipation of the proton gradient leads to the loss of intracellular potassium, sodium and magnesium and a decrease in the dry cell mass and intracellular amino acid pools.

Amphotericin B is used to treat systemic infections. The drug is administered intravenously as it is poorly adsorbed from the gastrointestinal tract, and topical application does not result in detectable levels of amphotericin B in the systemic circulation (Sugar, 1986). The maximum dose is 1 mg/kg/day. Because of slow drug clearance an alternate day treatment schedule might be followed (Graybill and Craven, 1983). Its biological action is essentially the same as Nystatin: metabolic disruption and cell death are secondary consequences of metabolic alterations (Hammond et al., 1974). Odds (1979) listed several of the side effects, including flushing, convulsions, chills and fevers. Antifungal resistance is not a recognized problem in patients

(Kerridge and Nicholas, 1986).

5.2 5-Fluorocytosine

5-Fluorocytosine, the fluorinated pyrimidine, inhibits growth of yeasts and many fungi. Since it is well adsorbed by the gut it may be given orally, and distributes well in the body. It is the only antifungal in clinical use where the occurrence of resistance poses a serious problem (Kerridge and Nicholas, 1986).

The spectrum of 5-fluorocytosine is limited as an antifungal. However, activity is best in yeast species, including Candida (Vanden Bossche et al., 1987). Polak and Grenson (1973) described the mode of action. The compound is transported to the yeast cytoplasm via cytosine permease, where it is deaminated to 5-fluorouracil which is then phosphorylated for incorporation into cellular RNA; thus protein synthesis is disrupted.

5.3 Ergosterol biosynthesis inhibitors

Ergosterol biosynthesis inhibiting antifungals constitute the most important group of compounds developed for the control of fungal diseases in plants, animals and man.

5.3.1 Azole antifungals

An extensive number of imidazole and triazole antifungal agents have been synthesised and developed. Most of the currently available imidazoles and the triazole, terconazole, are mainly for topical treatment.

Ketoconazole was the first azole derivative orally active against yeasts, dermatophytes and dimorphic fungi (Van Cutsem et al., 1987). It is also available for topical treatment. Itraconazole has been used for the treatment of acute vaginal candidosis. Vanden Bossche et al. (1987) reported that this azole has to be given with or immediately after a meal; adsorption may be reduced in cases of impaired gastric activity, and after evaluation in 3000 patients itraconazole is well tolerated. Azoles act to inhibit the cytochrome P-450 dependent 14 α-demethylation of lanosterol with consequently, uncoordinated synthesis of chitin, disruption of intracellular enzymes and cell membrane disruption (Vanden Bossche et al., 1987). Since minute amounts of ergosterol are needed to stimulate cell proliferation, the depletion of ergosterol, reacted at very low concentrations of azole derivatives, will also contribute to their antifungal activity.

Clotrimazole was the first substituted imidazole to be released as an antifungal drug, and miconazole was the second to have been accepted for clinical use. Miconazole inhibits fungal growth and growth of some Gram

positive bacteria. The biochemical effects of miconazole resemble clotrimazole; topical creams are now in wide usage.

Since the introduction of the imidazole antifungals in the late sixties and their use in millions of patients, only a few isolates of C. albicans have shown resistance. With ketoconazole, 4 isolates of C. albicans have been found to be much less sensitive to several azole antifungals; two were isolated from American patients (Horsburgh and Kirkpatrick, 1983) with chronic mucocutaneous candidosis and the other two were isolated from British chronic mucocutaneous candidosis patients, (Johnson et al., 1984; Ryley et al., 1984). These patients relapsed following prolonged treatment with ketoconazole.

5.3.2 Allylamines

The two allylamines, naftifine and terbafine are antifungals, of which the former is used topically for superficial mycoses and the latter for topical and oral treatment (Ryder et al., 1986).

Naftifine exhibits high activity against Trichophyton and against Aspergillus species. Terbinafine is a derivative of naftifine with excellent activity against dematophytes (Petranyi et al., 1984). Both these allylamines interfere with ergosterol synthesis as squalene epoxidase inhibitors (Vanden Bossche et al., 1987). Both compounds have a naphthalene moiety, which

may indicate that this moiety is involved in binding to the squalene epoxidase system.

6 PATHOGENICITY

6.1 Pathogenicity of different Candida species

The pathogenic Candida species can be listed in probable order of decreasing virulence: C. albicans, C. tropicalis, C. stellatoidea (now the same species as C. albicans), C. parapsilosis, C. pseudotropicalis, C. krusei, C. guillermondii (Odds, 1979). C. lusitaniae, C. glabrata and C. viswanthii were not mentioned in this report. There have been few studies comparing the pathogenicity of the various species of Candida under well controlled and reproducible experimental conditions. Only C. albicans, C. tropicalis and C. viswanthii were shown to be pathogenic on intravenous challenge (Bistoni et al., 1984), in decreasing order of pathogenicity. This latter study indicated that strong immunodepression (by cyclophosphamide) did not affect the susceptibility of mice to Candida species with low or no pathogenicity for mice, yet greatly increased susceptibility to Candida species with pathogenicity for an unmodified host.

Allen and Beck (1983) reported strain related differences in the pathogenicity of C. albicans in the rat mucosae.

Pope and Cole (1982) compared gastrointestinal colonization and systemic spread by C. albicans and the non-pathogenic C. guilliermondii and S. cerevisiae. Scanning electron microscopic investigations of the gastrointestinal tracts of animals receiving C. guilliermondii or S. cerevisiae were devoid of adherent yeasts. Barrett-Bee et al. (1985) compared phospholipase activity, adherence and pathogenicity in C. albicans, C. parapsilosis and S. cerevisiae. C. albicans isolates which were highly adherent were the most pathogenic in mice and had high phospholipase activities. By contrast, C. parapsilosis and S. cerevisiae were not very adherent, did not kill mice and had lower activities of phospholipase.

Wingard et al. (1979) reported C. tropicalis as the aetiologic agent in 15 of 18 cases of disseminated candidosis in patients undergoing treatment for haematologic malignamies or receiving bone marrow transplants.

Morgan et al. (1984), reported that in the case of C. pseudotropicalis fungaemia in the immunocompromised host, the source of infection was the urinary tract. Finally, Marrie et al. (1984) reported C. parapsilosis endocarditis; thus it can be concluded that several Candida species are pathogenic to man.

6.2 Course of infection

During the course of systemic infection, organisms become widely disseminated in the blood and may be found in most tissues; lesions may be found in kidneys, lungs, liver, heart, spleen and other organs: the extent and severity depends on the size of the initial inoculum (Odds, 1979). The length of infection also depends upon the prescribed treatment and its efficacy. In the case described by Bandini et al. (1986) fever was followed by acute renal failure, jaundice, progressive loss of consciousness and then death, in the space of two weeks after bone marrow transplantation.

6.3 Host responses

A variety of protective mechanisms are active intra-orally. Epstein et al. (1984) and Smith (1985) have both reviewed this subject. When these host defence mechanisms act in concert, they represent a formidable barrier to candidosis. In the oral cavity there are a variety of non-specific host-defence mechanisms. The epithelium acts as a physical barrier; epithelium turnover contributes to defence. Outwith the oral cavity, the stratified squamous epithelium of the skin is not usually colonised by C. albicans but is an effective barrier to invasion. Regular sloughing off and replacement affect loss of attached organisms (Smith, 1985). Saliva functions by its diluting and washing

effect, and it also contains antimicrobial factors such as lysozyme and lactoferrin.

Lysozyme is present in the oral cavity; it originates from saliva, gingival crevice fluid and polymorphonuclear leukocytes. It can agglutinate and kill C. albicans in non-ionic solutions and increases the fungicidal activity of amphotericin B in culture media (Epstein et al., 1984). Salivary glycoproteins may form part of the host's non-specific defence mechanism; they may be similar to those on the host cell surface preventing adhesion to the mucosal epithelium. Lactoferrin has the ability to chelate iron from the oral environment and has antimicrobial activity at oral concentrations. Its acitivity is believed to be due to the binding of iron which decreases the amount of available iron required for growth (Kirkpatrick et al., 1971). Cell-mediated immunity may be active in host resistance to some forms of candidosis (Epstein et al., 1984) and has been shown to be significant in chronic mucocutaneous candidosis (Kirkpatrick et al., 1971).

In oral microbial interactions, competition and inhibition are important in limiting the establishment and overall growth of fungi. Bacterial flora may restrict, but not eliminate Candida. Endogenous bacterial flora may decrease colonization by competing for adherence sites on epithelial cells (Marrie and Costerton, 1981). Gnotobiotic mice are colonized by C.

albicans when challenged. This enhanced susceptibility is due to reduced inflammatory response, delayed cell-mediated immunity, lower levels of immunoglobulin, reduced chemotaxis and lack of competition for receptor sites.

Alterations in the microbial flora of healthy individuals can be associated with systemic illness, hormonal changes, use of corticosteriods and antibiotics (Rogers and Balish, 1980). Instances of vaginal candidosis increase in pregnancy, and may be associated with increased glycogen in vaginal secretions (Knight and Fletcher, 1971).

The binding of antibodies to antigens in general is known to activate the serum complement system, one of whose functions is to facilitate phagocytosis. Epstein et al. (1984) described anti-Candidal IgA antibodies in saliva that could inhibit attachment of C. albicans to epithelial cells. They further noted that salivary levels of IgA and other anti-Candidal antibodies were higher in subjects with candidosis than in non-infected controls.

The increasing incidence of vaginal candidosis has led to interest in the origin and role of anti-C. albicans IgA and IgG in human cervicovaginal secretions. Gough et al. (1984) measured the levels of anti-C. albicans IgA and IgG in the genital tract of secretions of serum of non-pregnant women with vaginal candidosis

relative to uninfected women. There was however, no significant difference between the mean levels of specific IgA or IgG in secretions from women from the two groups.

6.4 Determinants of virulence

Several virulence factors of Candida albicans have been suggested (Table 1).

6.4.1 Toxins

Information about C. albicans toxins is both limited and contradictory. Hasenclever and Mitchell (1963) supported the idea of a C. albicans endotoxin but could not isolate it. It is clear that no Candida species secretes into its culture medium any molecule with a biological activity/potency equivalent to a bacterial endotoxin (Odds, 1988).

Indirect evidence of toxin existence came from the cell-free experiments of Davies and Reeves (1971). However, Stanley and Hurley (1967) had already cast doubt on the use of cell-free experiments, as when they used Candida species with tissue culture, no indirect toxic effect was seen. Cutler et al. (1972) found that only cell-wall glycoproteins of C. albicans had toxic effects. However, cell-wall glycoproteins from C. albicans may be the attachment site for the yeasts to interact with epithelial cells. McCourtie and Douglas (1985) found that extracellular glycoproteins isolated from culture

Table 1 Postulated virulence factors of *Candida albicans*

Virulence factor	Reference
Adhesion to mucosal surfaces	Douglas, 1987
Production of hyphae	Odds, 1988
Secretion of hydrolytic enzymes	Barrett-Bee *et al.*, 1985

supernatants of C. albicans inhibited adhesion of yeasts to buccal epithelial cells when epithelial cells were pretreated with the extracellular glycoprotein. It has been shown that Candida hyphae liberate substances which inhibit attachment of hyphae to neutrophils (Diamond et al., 1980). Iwata (1976) also described a "canditoxin". The lack of confirmatory data would bring the existence of such a toxin into question.

6.4.2 Filamentation and phenotypic variability

The ability of C. albicans to produce hyphae has long been held to account for its relatively high pathogenicity (Louria and Brayton, 1964). The effects of filamentation within tissues appears to confer on C. albicans a heightened mechanical ability to resist phagocytosis and penetrate mammalian cells (Saltarelli et al., 1975). However, Cockayne and Odds (1984) suggested that hyphal forms of C. albicans are more susceptible to killing by human polymorphonuclear effectors than are yeast forms. This would seem to contradict the commonly held belief that hyphal forms of C. albicans are endowed with a greater invasiveness and resistance to host defences than the yeast form.

Several microbial pathogens are known to express variable antigenic components, for example, typanosomes, Neisseria meningitidis and E. coli. It is therefore possible that Candida can do something similar. Anderson and Soll (1987) noted that C. albicans switched from

white to opaque colony types. They thought it unlikely that such a carefully organized high frequency switch to the opaque phenotype was for any other purpose than pathogenesis.

6.4.3 Enzymes

Hydrolytic enzyme activities expressed at the cell surface of microbial pathogens are always theoretically capable of damage to host cells *in vitro*. With *C. albicans* there are two main candidates: a proteinase and a phospholipase. Acid proteinases are secreted by most strains of *C. albicans*, *C. tropicalis* and *C. parapsilosis*; this reflects the sequence of virulence of these species in man (Rüchel, 1984; MacDonald, 1984). An inducible proteinase secreted by *C. albicans* is likely to be a virulence factor of the yeast. Budtz-Jörgensen (1971) indicated that clinical isolates of other *Candida* species secreted proteinases, while Staib (1965) reported that not all strains of *C. albicans* release an acid proteinase.

The location of *Candida* proteinase around the fungal cells invading host tissue suggests the importance of the enzymes in the pathogenesis of *Candida* infections (MacDonald and Odds, 1980).

Tsuboi *et al.* (1989) suggested that extracellular proteinase from *C. albicans* was activated by the acidification of the microenvironment close to the organism.

In the buffered milieu of the host, above pH 7.0, little acid proteinase activity can be expected. Candida acid proteinase can however be traced in the serum of mice in the early stages of experimental candidosis; such a circulating enzyme may become active in acidic pockets of damaged kidney. Muscle tenderness, an early sign of disseminated candidosis (Jacobs et al., 1980), may reflect an effect of acid proteinases. Evidence that the inducible acid proteinase of C. albicans may be one of its attributes of virulence comes from MacDonald and Odds (1983) and Kwon-Chung et al. (1985). They showed significant reductions in mouse lethality of mutant strains deficient in secretion of the enzymes. In the latter case a proteinase-secreting "revertant" isolated from an infected mouse, was shown to have the same virulence as the parent strain.

C. albicans produces phospholipases which may be associated with the pathogenicity of the fungus. In stationary-phase cells, Pugh and Cawson (1975) demonstrated the localization of the phospholipase activities in cells and culture medium by a cytochemical method.

Phospholipase A and lysophospholipase activities have been located in yeast cells and hyphae of C. albicans infecting chick chorioallantoic membranes using cytochemical techniques (Pugh and Cawson, 1977). Barratt-Bee et al. (1985) measured phospholipase A and

lysophospholipase activities in culture fluids. They showed correlation between phospholipase activity, pathogenicity and adherence to epithelial cells. Non-pathogenic yeasts and some C. albicans isolates which did not adhere, and did not kill mice, had lowered phospholipase activity.

7 ADHERENCE OF CANDIDA TO SURFACES

7.1 Importance of adherence

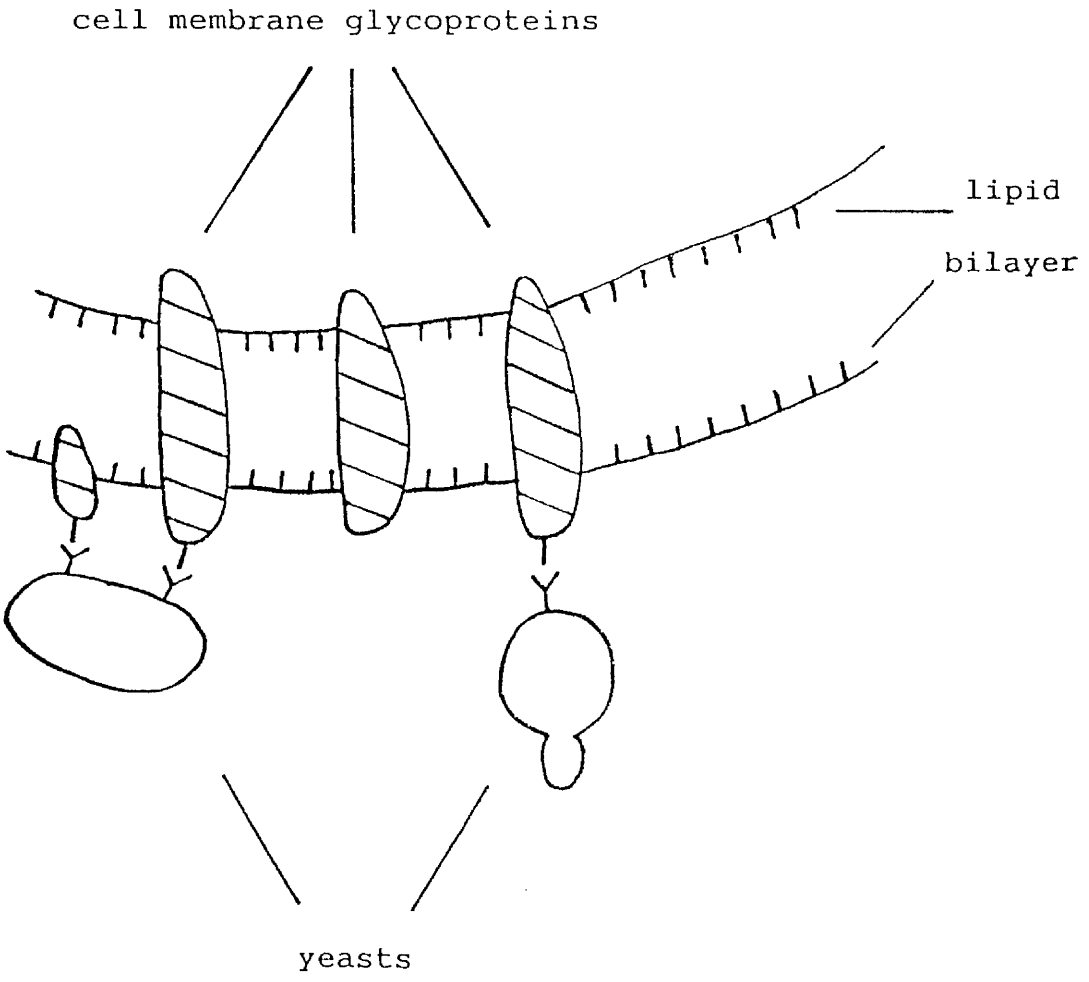
Colonization of mucosal surfaces by Candida species depends on the organisms' ability to adhere. Microorganisms are continually lost by the process of desquamation or "sloughing off" of surface epithelial cells. Organisms must adhere to the newly presented surface for effective colonization. This is one of the host defences against infection. With Candida species, which are opportunistic pathogens, colonization can be effected without clinical infection. The crucial role of adhesion in the pathogenesis of microbial infections is well recognized. Predominantly, reviews have focused on bacterial adherence (Wardell et al., 1983; Jones and Isaacson, 1983; Feingold, 1986). However the subject of yeast adherence is now being studied intensively and has been reviewed on several occasions (Douglas, 1985; Lee and King, 1983; Rotrosen et al., 1986; and Douglas, 1987).

Microbial attachment to surfaces is of wide ranging interest to physical chemists, engineers, dentists, plant pathologists and microbiologists. In recent years there has been a growing volume of literature produced on yeast adhesion and in particular of C. albicans attachment to epithelial surfaces. Non-specific microbial adhesion occurs with organisms capable of adhering to many different types of surface. Specific adhesion involves interactions between complementary molecules on the microbial and attached surfaces.

Initial loose docking is followed by permanent attachment possibly involving specific bonds between complementary molecules (see Figure 3). Using various sugars and lectins, inhibition assays have suggested that various glycosides act as receptors on mucosal epithelial cells (Ofek et al., 1978); and this adhesion is the initial step in infection. Considerable progress has been made in developing methods of inhibition of adherence of pathogens to mucosal surfaces (Beachey, 1981).

The theory of Derjaguin, Landau, Ver and Overbeck (DLVO Theory) has been useful in interpreting the interaction between two negatively charged bodies (Ho, 1986). The DLVO theory states that as two rigid bodies of like charge approach each other, they are subject to forces of attraction and repulsion, which are additive but which vary with distance (Isaacson, 1983). For

Figure 3 Adhesion of yeast to epithelial cells. Adhesion of yeast by specific adhesins (Y) to complementary receptor (|) on the host cell membrane


cell membrane glycoproteins
lipid
bilayer
yeasts

microorganisms to attach to mucosal surfaces, net repulsion must be overcome. Ho (1986) summarized the factors influencing whether adhesion should take place.

Extracellular surface polymers seem to have a role in mediating attachment. Marrie et al. (1983) saw extensive extracellular matrix formation in a very wide range of organisms including C. albicans. Ayers et al. (1979) were able to correlate the production of extracellular polysaccharide by bacteria with their ability to cause disease in host inoculation tests.

McCourtie and Douglas (1984) correlated adherence to acrylic and buccal epithelial cells with C. albicans cell surface composition and virulence. The C. albicans fibrillar floccular layer may represent something analogous to bacterial fimbriae, whose importance in adhesion is well documented (Jones and Isaacson, 1983). In E. coli the pili or fimbriae appeared to be involved in the pathogenesis of infection (Guerina et al., 1983). Fimbriae have been reported in a variety of yeast species including C. albicans (Gardiner et al., 1982). The determination of the nature of the host cell receptors has involved the use of sugar analogues to inhibit adhesion, or the binding of receptors by lectins. Lectins of known carbohydrate specificity have been used to inhibit adhesion (Critchley and Douglas, 1987b).

7.2 Measurement of *in vitro* adherence

The first quantative determination of *Candida* adhesion was that of Liljemark and Gibbons (1973). Their procedure was modified by Kimura and Pearsall (1978) to yield an assay now used in several laboratories to determine the adhesion of *Candida* to epithelial cells. The final stage of determining the number of adherent yeasts is performed either using a visual count (Douglas *et al.*, 1981), a radiolabelling count (King *et al.*, 1980) or a Coulter count (Gorman *et al.*, 1986). The method described by Segal *et al.* (1982) only determines epithelial cells with a minimal number of yeasts attached.

7.3 Factors influencing adhesion

A wide variety of factors are thought to be likely modulators of the adhesion process and they have been categorized (Douglas, 1985; 1987) into three groups: yeast factors, host factors and environmental factors (see Table 2). A wide range of cells and surfaces to which *Candida* species adhere have been found. These include buccal epithelia, vaginal epithelia, corneocytes, murine gastrointestinal epithelia, vascular endothelia and platelet matrices. *Candida* species have also been found to adhere to foreign bodies within the human body such as catheters, contact lenses and dentures. Only a few of these will be considered.

Table 2 Factors influencing adherence of *Candida* to epithelial cells
(adapted from Douglas, 1985)

Yeast Factors
Concentration and viability
Phased temperature growth
Growth medium composition
Species and strain
Germ-tube formation
Epithelial Cell Factors
Cell type
Environmental Factors
Temperature and time of contact
pH
Bacteria

7.4 Adherence of C. albicans

7.4.1 Adherence of C. albicans to buccal epithelial cells

The adherence of C. albicans and other Candida species to buccal cells has been measured by several investigators (King et al., 1980; Macura et al., 1983; Ray et al., 1984 and Critchley and Douglas, 1985). C. albicans was found to be the most adherent species, whilst C. tropicalis and C. stellatoidea (now C. albicans) exhibited moderate adherence capabilities and C. parapsilosis was only slightly adherent to buccal cells. These studies suggest a relationship between the ability of different Candida species to adhere to epithelial cells and their capacity for colonizing and infecting mucosal surfaces. Strains of C. albicans from active infections showed enhanced adhesion after growth in a defined medium containing 500 mM galactose (Douglas et al., 1981). In response to this high sugar concentration these strains synthesize surface components which are responsible for enhanced adhesion.

Kimura and Pearsall (1980) reported that germinating yeasts were more adherent than non-germinating ones. Cox (1983) investigated the effect of pH on adherence of Candida albicans to buccal cells, and found no significant change in adherence between pH 4 to 11. Davidson et al. (1984) claimed that yeasts were more adherent to buccal cells from adults than those from

children. This was in contrast to the earlier data of Cox (1983) that adults gave adherence values similar to those from children; the results of this latter study suggested that receptors remain stable and are unaffected by age. C. albicans was more adherent to buccal cells from children with oral candidosis and oral colonization than to buccal cells from uninfected control children (Cox, 1983).

7.4.2 Adherence to vaginal epithelial cells

Adherence to vaginal epithelial cells is not markedly influenced by pH (King et al., 1980), salts or divalent cations, so adherence is unlikely to involve only electrostatic or ion-bridging bonds. Lee and King (1983) showed that Candida cell fragments contained the adherence factor; the integrity of yeast wall mannoproteins was important for adhesion.

Germinating yeasts were more adherent to vaginal cells than were non-germinating yeasts in in vitro experiments (Sobel et al., 1981). This study also found that there was higher adherence to vaginal cells at pH 6 than at pH 3 to 4 which is nearer the normal vaginal pH. Persi et al. (1985) found that adhesion to vaginal cells was affected by the strain of C. albicans used, the side of the epithelial cell exposed to the yeast, pH conditions and CO_2 levels within the assay mixture.

Segal et al. (1984) correlated the hormonal status of women and adherence to vaginal cells. They further

reported that vaginal cells from fecund women who were pregnant and/or diabetic had a greater propensity to bind C. albicans than did epithelial cells from oral contraceptive users and non-pregnant diabetic controls. Differences in candidal adhesion between vaginal and buccal epithelial cells are significant, as are differences between cell donors (Sobel et al., 1981).

7.4.3 Adherence to vascular endothelium

Deep seated infection by C. albicans is usually due to widespread dissemination of the organism via the bloodstream; to invade the parenchyma of various organs, the yeast must attach to vascular endothelium and penetrate the vessel wall.

Barnes et al. (1983) demonstrated that adhesion of C. albicans to renal endothelium is mediated by fibrils on the yeast surface. Klotz et al. (1983) investigated segments of pig aorta and found that the reason some tissues became infected more often than others was that the endothelium of some tissues contains more receptors than the endothelium of others. The penetration of endothelium by yeasts may be attributable to the action of exoenzymes of C. albicans. As destruction of the host cell progresses, the fungus penetrates deeper into the substance of the aorta. Formalin-killed yeasts adhered just as well as viable organisms. However, C. albicans treated with formalin or papain was incapable of adhering to vaginal epithelial cells but could localize in rabbit

kidneys to the same degree as untreated organisms, suggesting the mechanisms of adhesion to vaginal epithelial cells and renal endothelium are distinct (Lee and King, 1983).

7.4.4 Adherence to fibrin platelet matrices

Studies on the adherence of different Candida species to fibrin-platelet matrices formed in vitro (Maisch and Calderone, 1980) showed that C. albicans and C. stellatoidae were considerably more adherent than the other yeasts tested. Maisch and Calderone later (1981) suggested that surface mannan is an important determinant in the adherence of C. albicans to fibrin-platelet matrices formed in vitro on the endocardium of heart valves. Experiments in rabbits suggest that humoral antibody may protect against C. albicans endocarditis by inhibiting yeast adhesion in vivo (Scheld et al., 1983).

7.4.5 Adherence to denture acrylic

McCourtie and Douglas (1981) and McCourtie et al. (1985) found that adherence to acrylic in vitro was promoted by growing the yeasts to stationary phase in high concentrations of certain sugars as the carbon source. Adherence of yeasts could be further increased by pretreatment of the acrylic with crude mannoprotein isolated from culture supernatants (McCourtie and Douglas, 1985). Mannoprotein probably enhances adhesion by acting as a non-specific polymeric bridge. Divalent

cations and serum also promote adhesion to acrylic (Samaranayake et al., 1980).

7.4.6 Effect of commensal bacteria on adherence of C. albicans

Liljemark and Gibbons (1973) investigated the mechanism by which bacteria suppress colonization of mucosal surfaces by C. albicans. They found that twice as many yeasts attached to germ-free rat cells than to cells from conventional animals, suggesting that indigenous flora can interfere with adherence. Adherence of C. albicans and commensal bacteria to human epithelial surfaces has also been studied (King et al., 1980; Samaranayake and MacFarlane, 1982). Makrides and MacFarlane (1982) showed that although commensal bacteria like Escherichia coli and Klebsiella aerogenes enhanced candidal adherence, others such as Streptococcus sanguis and Strep. miteor decreased adherence.

Makrides and MacFarlane (1982) investigated the mechanism by which E. coli enhances adherence of C. albicans to epithelial cells. They indicated that increased candidal adherence due to E. coli is mediated by fimbriae on bacterial cells and mannose-like receptors on surfaces of both C. albicans and epithelial cells. Sobel et al. (1981) found that preincubation of vaginal epithelial cells with two vaginal isolates of Lactobacillus species significantly decreased yeast adhesion.

7.5 The yeast adhesin

Most experimental evidence indicates a role for mannoprotein in mediating yeast attachment to buccal and vaginal cells (Douglas, 1987). For example, Sandin et al. (1982) reported that preincubation of C. albicans with Concanavalin A, a lectin that binds α-linked D-mannose (or glucose) residues, decreased the adhesion to buccal epithelial cells by more than 80%. Adhesion can also be inhibited by pretreating C. albicans with proteolytic enzymes (Sobel et al., 1981) and reducing agents (Lee and King, 1983) such as β-mercaptoethanol and dithiothreitol. McCourtie and Douglas (1985) and Critchley and Douglas (1987a) inhibited adhesion by preincubation of buccal cells with mannoprotein obtained from culture supernatants of yeasts grown in a medium containing high concentrations of galactose. The mannoprotein nature of the adhesin has also been demonstrated in experiments with the antibiotic tunicamycin (Douglas and McCourtie, 1983). This antibiotic functions as an inhibitor of protein glycosylation; in yeasts it specifically inhibits synthesis of mannoprotein but not that of the other major wall components, glucan and chitin (Kuo and Lampen, 1974). Addition of tunicamycin to cultures of C. albicans in high-galactose medium at the end of exponential growth inhibited formation of the fibrillar layer. Consequently, the C. albicans showed decreased

adhesion to buccal cells (> 60%) compared to untreated controls. However yeasts grown in low-glucose medium and lacking in the fibrillar layer, were unaffected by tunicamycin treatment (Douglas and McCourtie, 1983). Extracellular material originating, at least partially, from a fibrillar layer on the yeast surface is thought to contain yeast adhesin as it inhibits adhesion. The specificity of this interaction can be demonstrated; mannoprotein isolated from one C. albicans (GDH 2023) failed to inhibit the adhesion of a second strain, namely C. albicans GDH 2346 (McCourtie and Douglas, 1985).

Adhesion of C. albicans to vaginal epithelial cells was severely inhibited by pretreating yeasts with a variety of proteolytic enzymes or with reducing agents such as β-mercaptoethanol or dithiothreitol (Lee and King, 1983). Critchley and Douglas (1987a) found that pretreatment of vaginal cells with crude mannoprotein from culture supernatants had similar results. Adhesion mechanisms in which proteinaceous adhesins interact with carbohydrates on animal cell surfaces have already been described (Jones and Isaacson, 1983). Involvement of the protein portion of the yeast mannoprotein in Candida adhesion would be analogous. By contrast, Segal et al. (1982) reported that chitin and chitin hydrolysate inhibited yeast adhesion to vaginal epithelial cells in vitro, yet chitin is thought to be concentrated in the inner layers of the yeast cell wall (Cassone et al.,

1973). Animal experiments by Lehrer et al. (1983) also showed that N-acetyl-D-glucosamine or an aqueous extract of chitin blocked yeast attachment to epithelial surfaces in vivo and thus precluded vaginal infection. These discrepancies remain unexplained.

Characterization of the mannoprotein adhesin involves identifying the fragment of the complex molecule that interacts with the epithelial cell. Pretreatment of crude adhesin with heat, dithiothreitol or proteolytic enzymes (except papain) either partially or completely destroys its ability to inhibit adherence. Protein-rich fractions obtained by endoglycosidase H digestion and fractionation on Concanavalin A columns, and used in inhibition assays indicated that the protein-rich fraction inhibits adhesion more than the carbohydrate-rich fraction. These results suggest that the major interaction between yeasts and epithelial cells is one involving a protein adhesin (Critchley and Douglas, 1987a). This would be analogous with the situation in many Gram negative bacteria (Jones and Isaacson, 1983).

7.6 The epithelial cell receptor

Sugars used to block yeast adhesion in hapten inhibition tests are those present on host cell surfaces in glycoproteins and glycolipids (Sharon et al., 1981). The plasma membrane of all animal cell surfaces consists

of a lipid bilayer and proteins. The composition and organization of the membrane has been reviewed by Ofek et al. (1985). In isolation these tests can provide equivocal results (Douglas, 1987); with Candida, mannose-containing components on both cell surfaces may participate in adhesion. Furthermore, some sugars may be rapidly metabolized during the assay by the microorganism under investigation, C. albicans, and this may produce spurious results. This would partially explain why sugar inhibition tests with C. albicans have yielded a variety of contradictory results. L-fucose (Sobel et al., 1981), amino sugars (Segal et al., 1982) and D-mannose (Sandin et al., 1982) have all been reported to be the major inhibitor of Candida adhesion and therefore the likely receptor determinant. Other investigations have failed to identify inhibition with any sugar (Reinhart et al., 1985), although a full range of sugars known to be constituents of epithelial cell membranes was not employed. The experiments referred to above would indicate that different strains of C. albicans bind to different glycoside receptors.

L-fucose is commonly found on the epithelial cell membrane of the rabbit gut and has been shown to function as a receptor for Vibrio cholerae (Jones and Freter, 1976). By use of experiments involving Concanavalin A, Sandin et al. (1982) concluded that mannose-containing moieties on the surface of C. albicans and buccal cells

would mediate adherence. Critchley and Douglas (1987b) utilized a wide range of lectins and sugars and concluded that depending on the strain of C. albicans, glycosides containing L-fucose, N-acetyl-glucosamine, or D-mannose may all function as epithelial receptors for the yeast. Douglas (1987) tabulated the inhibition of adherence of different strains of C. albicans to epithelial cells by sugars, and concluded that fucose-containing receptors may be commonly required for Candida adherence. Additional mechanisms may also function, since introduction of L-fucose into adherence assay mixtures only partially inhibits adhesion. Crude mannoprotein from C. albicans has been shown to contain "lectin-like" proteins capable of binding L-fucose, D-mannose and N-acetyl-D-glucosamine. The protein varies from one strain to another (Critchley and Douglas, 1987b). Thus the relative abundance of these proteins, and in particular their stereochemistry, determines the receptor specificity of different C. albicans strains (Douglas, 1987).

8 ABO, LEWIS AND RELATED BLOOD GROUP ANTIGENS

8.1 The ABO blood group

The first blood group system to be discovered in man was the ABO system by Landsteiner in 1900, cited by Green (1989). Clinically, this is the most important system.

Over six hundred other blood group antigens have been described on the red cell membrane. The membrane of the red blood cell is formed from a phospholipid bilayer containing glycolipids, proteins and glycosphingolipids (Gratzer, 1981). Bird (1983) defined blood groups as inherited differences in antigens of tissues, predominantly used with red cells, but also on platelets, tissues and various cell surfaces. The A and B blood group antigens are major histocompatibility antigens (Joysey et al., 1977) and can cause graft and transplantation rejection of various organs and tissues. A, B and H determinants are found in saliva, and most body secretions. In man, various receptor properties including specific interactions with antibodies, viruses, toxins and pharmacological agents, have been attributed to the ABH determinants (Green, 1989).

Figure 4 shows, in a simplified form, the genetic pathway for the ABO and Hh systems. The H gene product converts a precursor to H substance. This is the substrate for the ABO gene-specified transferase to catalyse the addition of the blood group sugar (Watkins et al., 1959). In hh individuals, often referred to as Bombay phenotype, there is no ability to convert the original precursor to the H active structure. Consequently the addition of A and B specific sugars (N-acetyl-D-glucosamine and D-galactose) is impossible (Green, 1989). Additionally, Rege et al. (1963)

Figure 4 Representation of the structures proposed for the carbohydrate chains in the precursor glycoprotein and the additions to these chains controlled by the He, Le, A and B genes.

Gal ---------- D-Galactose

GalNAc ---------- N-Acetyl-D-Galactosamine

Fuc ---------- L-Fucose

GNAc ---------- N-Acetyl-D-Glucosamine

Genes	Structure Formed	Chain	Specificity
Unidentified genes giving precursor	Galβ1,3GNAcβ1,3Galβ1,3GalNAc	1	-
	Galβ1,4GNAcβ1,3Galβ1,3GalNAc	2	Type XIV
H gene (addition of L-fucose to precursor	Galβ1,3GNAcβ1,3Galβ1,3GalNAc | |α1,2 Fuc	1	H
	Galβ1,4GNAcβ1,3Galβ1,3GalNAc | |α1,2 Fuc	2	H
Le gene (addition of L-fucose to precursor)	Galβ1,3GNAcβ1,3Galβ1,3GalNAc | |α1,4 Fuc	1	Le^a
	Galβ1,4GNAcβ1,3Galβ1,3GalNAc	2	Type XIV
H and Le genes	Galβ1,3GNAcβ1,3Galβ1,3GalNAc | | |α1,2 |α1,2 Fuc Fuc	1	Le^b
	Galβ1,4GNAcβ1,3Galβ1,3GalNAc | |α1,2 Fuc	2	H
A gene (addition of GalNAc to H-active chains)	GalNAcα1,3Gal (β1,3 or 1,4) | |α1,2 Fuc	1 and 2	A
B gene (addition of Gal to H-active chains)	Galα1,3Gal (β1,3 or 1,4) | |α1,2 Fuc	1 and 2	B

described the structure of H, A and B type 1 and type 2 chains. On the red cell, all ABH structures are synthesized on type 2 chains, up to 80% of these chains being bound to protein and some 20% being glycolipids. In secretions ABH structures are found as glycoprotein on both chain types.

8.2 Se, the secretor gene

An additional gene, Se is required by the 1-2 fucosyl transferase (specified by the H gene) to express itself in body secretions. The mechanism is not understood. There is evidence (Oriol et al., 1986) that the Se and H genes are two distinct but very closely related structural genes each coding for a distinct α-2-L-fucosyltransferase, one reacting preferentially with the type 1 precursor chain and the other reacting preferentially with type 2 precursor chains. The Se and se alleles segregate independently of the ABO and Hh genes. Se is dominant, therefore only sese individuals are non-secretors.

8.3 The Lewis system

The blood group antigens of the Lewis (Le) system differ from those of other systems in that they are not synthesized on the red cell but are present in plasma and in body secretions such as saliva. The Le-a and Le-b antigens are not antithelical i.e. they are not produced

by two allelic genes at a single locus (Green, 1989). Le and le are inherited independently of the ABO, Hh and Sese genes (Race and Sanger, 1975). However they are very closely related (Grubb, 1948). Lewis antigens have been reported, presumably in the specifically adsorbed form, on various epithelial tissues (Lemieux et al., 1981). Saliva from individuals whose red cell type is Le(a+b-) contains Lewis-a substance, but not Le-b substance. With the red cell type Le(a-b+), Lewis-b is found along with some Lewis-a. Individuals that are Le(a-b-) have neither substance, as the presence of Lewis substances in the saliva is not dependent on the presence of an Se (secretor) gene. Generally, however, secretors of blood group antigens are of Lewis-b type and non-secretors are Lewis-a.

8.4 Blood groups and infectious diseases

Correlations between ABO blood group and susceptibility to infections have been reported (Mourant et al., 1978; Deresinski et al., 1979) and blood group determinants like L-fucose have been shown to act as microbial receptors (Jones and Freter, 1976). ABO blood group antigens are not solely located on erythrocytes but are present on the cell surface of many cells including mucosal epithelia (Mollicone et al., 1985). Blackwell et al. (1986) suggested that non-secretion of blood group substances is associated with increasing susceptibility

to oral infection with C. albicans. They recently reported that non-secretion of ABO blood group antigens was associated with susceptibility to superficial fungal infections and also diabetes mellitus (Blackwell et al., 1990). This study only partially agreed with the earlier and more convincing data of Burford-Mason et al. (1988) which indicated that in healthy subjects, blood group O and non-secretion of blood group antigens were separate and cumulative risk factors for oral carriage of C. albicans. The study of Blackwell et al. (1990) concluded that there was no association between ABO blood group and carriage. May et al. (1989) suggested that the Lewis-a antigen may be one of the receptors for some yeast strains, as the binding of C. albicans to epithelial cells from a non-secretor was inhibited by pretreating with anti-Lewis-a, but this effect was not observed with epithelial cells from a secretor.

9 OBJECTS OF RESEARCH

The mechanism of adherence of Candida albicans to mucosal surfaces has yet to be determined. It is known that yeast adherence involves specific interactions between fungal adhesins and epithelial cell surface receptors. Previous data have shown that the fibrillar mannoprotein layer, produced when the organism is grown in high concentrations of galactose, contains the proteinaceous yeast adhesin. The aims of this project

were

(i) to characterize the adhesin further using chromatographic and electrophoretic techniques, and to identify by chemical and enzymic dissection of the molecule, the minimum structure required for adhesion inhibition.

(ii) to characterize the epithelial receptors involved in Candida adherence using sugars, lectins, and saliva to inhibit adherence.

This information may lead to the development of procedures to prevent infection.

MATERIALS AND METHODS

1 ORGANISMS

Four species of Candida were used in this study. Details of their sites of isolation are given in Table 3. Of the thirteen strains of C. albicans, strains 904, 905, 910 and 911 were isolated from a single patient but were shown by biotyping to be different.

The organisms were maintained on slopes of Sabouraud dextrose agar and subcultured monthly. Every two months cultures were replaced by new ones freshly grown from freeze-dried stocks. The identity of each isolate was checked using conventional techniques i.e. germ-tube formation and sugar assimilation tests.

2 GROWTH CONDITIONS

In all experiments, organisms were exclusively in the budding yeast phase.

2.1 For freeze-drying

For freeze-drying, yeasts were grown in yeast nitrogen base (Difco) containing 500 mM sucrose. Batches of medium (50 ml, in 250 ml Erlenmeyer flasks) were inoculated with overnight yeast cultures (5 ml) and incubated at 37°C in an orbital shaker operating at 150 rpm. Cells were harvested after 24 h (MSE bench centrifuge: 5 min, 1200 g) and washed in sterile 0.15 M phosphate-buffered saline (pH 7.2; PBS). Yeast cells

Table 3 Origin of *Candida* isolates used in this study

Species	Strain	Source	Type of infection	Site of isolation
C. albicans	GDH 2346	Glasgow Dental Hospital	Denture stomatitis	Oral cavity
	GDH 2023	"	"	"
	MRL 3153	Mycological Reference Laboratory	———	———
	GRI 681	Glasgow Royal Infirmary	None	Cervical smear
	GRI 682	"	"	"
	Outbreak	The London Hospital	Systemic candidosis	Oral cavity
C. albicans	RDC 902	Royal Dental College, Copenhagen	Erythroleukoplakia	"
	904[1]	"	Nodular leukoplakia	"
	905[1]	"	None	"
	906	"	None	"
	910[1]	"	Nodular leukoplakia	"
	911[1]	"	None	"
C. glabrata	RDC 909	"	Erythroleukoplakia	"
C. parapsilosis	RDC 912	"	"	"
C. lusitaniae	RDC 936	"	"	"
C. lusitaniae	RDC 937	"	None	"

[1] All four strains isolated from one patient

were resuspended in a small volume of 2% (w/v) sterile skimmed milk and a drop of suspension was added to a sterile glass ampoule. Ampoules were frozen before being dried in a centrifugal freeze-drier (Edwards High Vacuum Ltd. Sussex).

2.2 For adherence assays and determination of cell-surface hydrophobicity

Batches of medium (50 ml, in 250 ml Erlenmeyer flasks) containing yeast nitrogen base (YNB) and either 50 mM glucose, 500 mM sucrose or 500 mM galactose were inoculated from stock cultures and incubated overnight at 37°C on an orbital shaker operating at 150 rpm. The overnight culture (5 ml) was used to inoculate the same medium (50 ml) containing the same carbon source as before. The culture was incubated at 37°C on an orbital shaker at 150 rpm for 24 h so that the yeast cells reached the stationary phase of growth. Cells were harvested by centrifugation (MSE bench centrifuge: 5 min, 1200 g) and washed twice in 0.15 M PBS (pH 7.2).

2.3 For isolation of extracellular polymeric material

Yeasts were grown in YNB medium containg 500 mM galactose as the carbon source. Batches of medium (500 ml, in 2 l Erlenmeyer flasks) were inoculated with 50 ml of an overnight culture and incubated at 37°C on an orbital shaker operating at 150 rpm for 5 d.

3 ADHERENCE TO HUMAN BUCCAL EPITHELIAL CELLS

3.1 The yeast adherence assay

3.1.1 Collection and preparation of buccal epithelial cells

Buccal epithelial cells were collected from the appropriate (see Table 4) donor by gently swabbing the inside of the cheeks with a sterile swab. The swab was agitated in PBS (10 ml) in a universal bottle. Epithelial cells were harvested by centrifugation (MSE bench centrifuge: 5 min, 1200 g) and washed twice in PBS to remove loosely-bound microorganisms. After the second wash, epithelial cells were standardized using an improved Neubauer haemocytometer to a concentration of 1 x 10^5 cells ml^{-1} in PBS. Buccal cells were always collected between 8.30am and 10.30am to minimize variability. Details of the buccal cell donors are shown in Table 4. The criteria for all donors are listed in Table 5. These criteria were laid down so as to minimize variation between donors. Healthy individuals in the age range of 22-38 years were used in all but one of the assays. All were non-smokers and had had no recent antibiotic therapy. Each individual's buccal cells were screened for the carriage of Candida prior to their use in adherence assays.

Table 4 ABO and Lewis blood group, and secretor status of individual buccal epithelial cell donors

Donor	ABO group	Lewis a/ Lewis b group	Secretor/ non secretor	Sex (M or F)
F.D.T	A	Lewis a	Non secretor	M
H.S.	A	Lewis b	Secretor	M
A.E.	O	Lewis a	Non secretor	M
F.F.C.	O	Lewis b	Secretor	M
E.A.	O	Lewis b	Secretor	F
J.H.R.	A	Lewis a	Non secretor	F

ABO and Lewis blood groups, and secretor status were determined as described in Sections 5.1, 5.2 and 5.3 respectively.

Table 5 Criteria for buccal cell donors

All healthy individuals
Age 22-38
Non-smokers
No recent antibiotic therapy
No apparent carriage of Candida
Buccal cells collected at the same time of day

Criteria applied to all buccal cell donors except one who was more than 60 years old.

3.1.2 Preparation of yeast cells

After the second wash of the yeasts in PBS, yeast suspensions were standardized using an improved Neubauer haemocytometer to a final yeast concentration of 1×10^7 cells ml^{-1} in PBS. In certain experiments different concentrations were used as indicated separately.

3.1.3 Adherence assay

The method used was similar to that described by Douglas et al. (1981). Epithelial and yeast cell suspensions were vortexed using a whirlimixer before being used in adherence assays. Standardized suspensions of buccal epithelial cells (1×10^5 cells ml^{-1} in PBS; 0.1 ml) and yeasts (1×10^7 cells ml^{-1}; 0.1 ml) were mixed in small screw-capped bottles and incubated at 37°C, with gentle shaking, for 45 min. After incubation, 3 ml of PBS was added to each bottle to stop any further attachment. The epithelial cells were collected on polycarbonate filters (12 μm pore size; 25 mm diameter; Nuclepore Corp., Pleasanton, CA) and washed with PBS (25 ml) to remove unattached yeasts. The washed filters were placed on labelled slides and air dried. Epithelial cells on the filters were fixed with absolute alcohol and acetone (1:1) for 5 secs. and stained using the Gram procedure. After drying, filters were mounted under coverslips using DPX (BDH Chemicals Ltd.). The numbers of adherent yeasts on each of 100 epithelial cells were counted on every filter. Triplicate filters were

prepared for each assay. All adherence values quoted represent mean figures from 3 independent assays, except where indicated when two independent assays were used.

3.2 Use of inhibitors to block *in vitro* adherence

3.2.1 Extracellular polymeric material (EPM)

Epithelial cell suspensions (1 ml, containing 1 x 10^5 cells ml^{-1}) were centrifuged in an MSE bench centrifuge at 5000 g for 5 min and the supernatants discarded. Crude EPM samples (10 mg ml^{-1}; 1 ml) were added to the cell pellets and the cells were mixed using a whirlimixer. Epithelial cells were incubated with the EPM solution for 30 min at 37°C in an orbital shaker operating at 150 rpm. After this treatment, epithelial cells were recovered by centrifugation, resuspended in PBS (1 ml) and used in adherence assays.

3.2.2 Lectins

Ulex europaeus lectin (Anti H) (Inverclyde Biologicals, Barrhead) was used in saline at a concentration (5% v/v) that just gave positive agglutination of Group O red blood cells.

Epithelial cells (1 ml, containing 1 x 10^5 cells ml^{-1}) were harvested in a MSE bench centrifuge at 5000 g for 5 min. Cells were suspended in lectin solution (1 ml) and incubated at 37°C for 30 min on an orbital shaker operating at 150 rpm. After incubation, epithelial cells

were harvested and resuspended in PBS (1 ml). The lectin-pretreated epithelial cells were used in adherence assays.

3.2.3 Antisera

Various monoclonal antisera raised against different blood group antigens were used. Anti-A (kindly supplied by Mr J. Keary, Blood Bank, Glasgow Royal Infirmary), anti-Lewis a and anti-Lewis b (kindly supplied by Dr R.H. Fraser, West of Scotland Blood Transfusion Service, Law Hospital, Carluke) sera were all used to pretreat epithelial cells. Cells (1 ml, containing 1 x 10^5 cells ml^{-1}) were harvested using an MSE bench centrifuge at 5000 g for 5 min. Appropriate antiserum (1 ml) was added to the cell pellet and the cells were gently resuspended. The suspension was incubated at 37°C for 30 min on an orbital shaker operating at 150 rpm. After incubation, epithelial cells were harvested and resuspended in PBS (1 ml). The antiserum-pretreated epithelial cells were then used in adherence assays.

3.2.4 Saliva

Saliva from each subject (2 ml) was collected in a glass test-tube, boiled for 20 min and then centrifuged at 5000 g for 10 min. The supernatant was either stored frozen at -20°C until tested, or used immediately. Buccal epithelial cells (1 ml, containing 1 x 10^5 cells ml^{-1}) and yeast cells (1 ml, containing 1 x 10^7 cells

ml^{-1}) were harvested using an MSE bench centrifuge at 5000 g for 5 min. The cell pellets were resuspended in saliva (50% in PBS), mixed and incubated at 37°C for 45 min on an orbital shaker operating at 150 rpm.

3.2.5 Sugars

All sugars, L-fucose, D-mannose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine (all from Sigma), and D-galactose (Koch-Light) were used at a final concentration of 25 mg ml^{-1} in PBS. Epithelial cells (1 ml, containing 1 x 10^5 cells ml^{-1}) were centrifuged using an MSE bench centrifuge at 5000 g for 5 min and the supernatant removed. The pellet was resuspended in the appropriate sugar solution (1 ml, containing 25 mg ml^{-1}).

Yeast cells (1 ml, containing 1 x 10^7 cells ml^{-1}) were centrifuged using an MSE bench centrifuge at 5000 g for 5 min. Sugar solution (1 ml) was added to the yeast pellet. All cells were thoroughly vortexed using a whirlimixer prior to use in adherence assays. Adherence assays were performed as described previously, using epithelial cells or yeasts suspended in sugar solutions.

3.3 Isolation and purification of yeast adhesin

3.3.1 Preparation and analysis of extracellular polymeric material (EPM)

3.3.1.1 Isolation of EPM from culture supernatants of Candida albicans: extracellular polymeric material (EPM) was prepared by freeze drying (i) dialysed culture supernatants, or (ii) ultrafiltered and dialysed culture supernatants. Batches of medium (500 ml in 2 l Erlenmeyer flasks) containing yeast nitrogen base (Difco) + 500 mM galactose were inoculated with overnight yeast cultures (50 ml) and incubated at 37°C for 5 d in an orbital shaker operating at 150 rpm. Yeasts were removed by centrifugation and the culture supernatant fluid was (i) dialysed at 4°C for one week against five changes (12 l each) of distilled water, or (ii) ultrafiltered in an Amicon DC5 Hollow Fibre Concentration System using a 5K filter and then dialysed at 4°C for 3 d against 5 changes (5 l each) of distilled water. The retentate (crude EPM) was freeze dried and weighed.

3.3.1.2 Biochemical analysis of EPM: carbohydrate was estimated by the method of Dubois et al. (1956) using mannose as a standard (see Appendix 1A). Protein was determined using the method of Lowry et al. (1951) with bovine serum albumin as a standard (see Appendix 1B). Phosphorus was estimated according to the method of Chen et al. (1956) using KH_2PO_4 as a standard (see Appendix

1C).

3.3.2 Isolation of "lectin-like" components from EPM

L-Fucose, D-mannose and N-acetyl-D-glucosamine (Sigma) were bound to an inert matrix using a procedure that involved swelling epoxy-activated Sepharose (1 g) in distilled water for 15 min, and then washing the gel on a sintered glass filter with more distilled water (100 ml). Ligand solution (50 mg ml^{-1} of sugar; 3 ml) was mixed with the gel suspension and incubated at 37°C for 16 h in a shaking water bath. Excess sugar was removed by washing with distilled water (100 ml), 0.1 M sodium bicarbonate buffer, pH 8.0 (100 ml) and 0.1 M sodium acetate buffer, pH 4.0 (100 ml). Any remaining reactive groups were blocked by treatment with 1 M ethanolamine (5ml) at 45°C for 9 h. The gel was finally washed with PBS and transferred to a small column (100 x 7 mm). EPM (200 mg ml^{-1} in PBS; 1 ml) was applied and the column eluted with PBS. The protein contents of the EPM solution and the eluate were determined by the Lowry method so that the percentage of EPM protein that bound to the gel could be calculated. Bound material was eluted with a solution of the appropriate sugar (25 mg ml^{-1}; 10 ml). The eluate was freeze dried, then reconstituted in 1 ml of water and applied to a Sephadex G-25 desalting column (Pharmacia; 5 ml). The desalting column was equilibrated with PBS and an A_{280} profile was

recorded for 0.5 ml fractions. Fractions constituting the protein peak were pooled and used in adhesion inhibition assays.

3.3.3 Chemical and enzymic treatments of EPM

In some experiments, crude EPM used to treat epithelial cells was processed as described in 3.3.3.1 and 3.3.3.2. In the others, the treatments described were used as pretreatments of EPM before its application to Synsorb affinity matrices.

3.3.3.1 Enzyme treatments: N-Glycanase (Genzyme; 250U ml^{-1}; 0.0024 ml) was used to treat EPM samples (100 mg ml^{-1} in 0.55 M Na_2HPO_4, pH 8.6; 0.05 ml) for 24 h at 37°C. The reaction was terminated using 0.1 M sodium citrate buffer, pH 4.4 (0.01 ml).

Papain (Sigma) was used at a concentration of 0.1 mg ml^{-1} in 0.01 M potassium phosphate buffer, pH 6.2 (0.016 ml). EPM (1 mg ml^{-1}; 1 ml) was incubated with this enzyme solution at 25°C for 30 min and then 0.05 ml of α_2 macroglobulin (2 mg ml^{-1}), an enzyme inhibitor, was added.

3.3.3.2 Alkali treatment: mild alkali treatment involved dissolving EPM (20 mg ml^{-1}) in NaOH (0.1 M; 1 ml) in a screw capped bottle; the solution was incubated at 30°C for 24 h and then neutralized with HCl solution (0.1 M; 1 ml) to give a final EPM concentration of 10 mg ml^{-1}.

Table 6 shows the final large scale purification protocol for the yeast adhesin. Digests of EPM prepared from each stage of the procedure were used as inhibitors of yeast adhesin. In some cases EPM was processed through merely the first stage in the procedure i.e. N-Glycanase degradation; in others EPM was given the full treatment protocol.

3.3.4 Isolation of purified adhesin by "Synsorb" affinity chromatography

Synsorbs (Chembiomed Ltd, Edmonton, Alberta, Canada) are specific affinity adsorbents for the isolation and purification or removal of biologically important materials. They are composed of synthetic carbohydrate structures of known specific composition covalently linked to an inert, insoluble matrix (i.e. immobilized oligosaccharides). Material adsorbing to the immobilized oligosaccharide can be removed in a highly purified form and the adsorbent regenerated by the elution of bound materials with high or low pH buffers.

Synsorb (0.05 g) of the four types shown in Table 7, was suspended in PBS (2 ml) in a Buchner flask and degassed using a vacuum pump. The Synsorb slurry was washed in a plastic, screw capped tube with 2 ml of PBS and allowed to settle. After this, the buffer level over the Synsorb was lowered to just above the surface of the Synsorb bed and EPM solution (10 mg ml^{-1}, 1 ml) or purified EPM digest (1 ml) was applied. The EPM and the

Table 6 Final purification protocol developed for the yeast adhesin

EPM

100 mg ml^{-1}; 1 ml in 0.55 M Na_2HPO_4, pH 8.6

+

N-Glycanase 250U ml^{-1}; 0.0096 ml

| 24 h at 37°C

V

Add 0.04 ml 0.1 M sodium citrate buffer, pH 4.4, to terminate reaction

| N-Glycanase ——>

V

Adjust EPM concentration to 50 mg ml^{-1} with 0.01 M potassium phosphate buffer, pH 6.2

+

Papain (0.1 mg ml^{-1}; 0.16 ml)

| 30 min at 25°C

V

Add α_2 macroglobulin (2 mg ml^{-1}; 0.5 ml)

| Papain ——> —> use in adherence assays

V

Add 1 ml NaOH (0.1 M)

| 24 h at 30°C

V

Add 1 ml HCl (0.1 M)

| Mild alkali ——>

V

Synsorb H-2 affinity adsorption

Affinity adsorption ——>

Table 7 Immobilized oligosaccharides bound to silica matrix (Synsorbs)

Oligosaccharide		Trivial Name
βGal(1-3)βGlcNAc | (1-4) αFuc	-	Lewis-a
αFuc(1-2)βGal(1-3)βGlcNAc | (1-4) αFuc	-	Lewis-b
αFuc(1-2)βGal(1-3)βGlcNAc	-	H-1
αFuc(1-2)βGal(1-4)βGlcNAc	-	H-2

Synsorb were gently rotated for 3 h at 4°C. The adsorption of EPM solution was halted by the addition of 1 ml of PBS, and the unbound material eluted using PBS until the A_{280} returned to 0.0. Bound material was then eluted by the addition of 1 ml aliquots of 1.5% NH_4OH in sterile saline for 15 min at 4°C until the A_{280} returned to 0.0. Bound samples were freeze-dried, reconstituted in water and desalted on a Sephadex G-25 (5 ml) desalting column, prior to incorporation in adherence assays. The adsorbent was re-equilibrated with PBS and then stored at 4°C in PBS containing 0.02% NaN_3 as a bacteriostat.

3.4 Determination of yeast cell surface hydrophobicity

The method of Sweet et al. (1987), using the volatile hydrocarbon xylene was followed. Suspensions of yeasts were adjusted to an A_{600} of 1.3(±0.1) measured in an SP 8-100 spectrophotometer (Pye Unicam).

To each of two 15 mm diameter glass test-tubes, 5 ml of the test suspension was added representing one sample and one control. In addition a sample and control were prepared on the suspending medium alone as spectrophotometer blanks. To each sample suspension was added 1 ml of xylene (Analar). The samples and controls were placed in a water bath at 37°C for 10 min to equilibrate, then vortexed in turn for 30 sec and returned to the water bath for 30 min to allow the immiscible xylene and aqueous phases to separate. The

lower aqueous layers were carefully removed using pipettes and transferred to fresh test-tubes. Any contaminating xylene that may have been carried over was removed by bubbling air through the suspensions for 1 min. Absorbances were measured as before following vortex mixing for 5 sec to disrupt and resuspend any aggregates that might have formed. Finally, the percentage reduction in absorbance of the test suspensions (with xylene) compared with the control suspensions (without xylene) was calculated. Each assay was performed in triplicate.

4 SDS-PAGE ELECTROPHORESIS

4.1 Gel preparation and electrophoresis

The procedures for preparing stock solutions are given in Appendix II. Gel plates were prepared as follows. To prevent staining artefacts the plates were washed with detergent, thoroughly rinsed with distilled water and wiped clean with paper tissues dipped in absolute alcohol. Two plates were taped together along three sides and baked for 30 min at 80°C to seal the tape. Rinsed plastic gloves were worn at all times to prevent fingerprints.

The gels were prepared as follows. The separating gel solutions containing 12.5% acrylamide, 1.5% Tris-HCl (pH 8.9), 0.4% SDS, 0.025% TEMED and 0.8% ammonium

persulphate, were poured into the gel plates to about 4.0 cm from the top. Then either saturated butan-1-ol or absolute alcohol was used to overlay the separating gel, which was left for about 20 min to polymerize. After polymerization, the overlay solution was removed and the gap between the top of the gel plates and the top of the gel was washed thoroughly with distilled water. Stacking gel solution containing 4.5% acrylamide, 0.5 M Tris-HCl (pH 6.8), 0.4% SDS, 0.025% TEMED and 0.08% ammonium persulphate, was used to fill the gel plates almost to the top. Sample combs were introduced into the stacking solution and any remaining spaces were filled with the stacking gel solution. This was allowed to polymerize and the combs were then removed and the gel plates were dipped in running buffer to avoid drying out. Gels were also prepared with 15% acrylamide in the separating gel. Tris-glycine running buffer (pH 8.3) containing 0.1% SDS was used to fill the lower electrode tank. The sealing tape was removed from the bottom of the gel plates, and the plates were inserted into the top electrode tank. The upper electrode was filled with running buffer. Samples of EPM (10 mg ml^{-1}; 20 μl) treated with solubilizing buffer (see Appendix II) were carefully pipetted into the sample wells. Gels were run at a constant current at 10°C in a Pharmacia GE-24LS tank (20 mA per gel) for approximately 3.5 h. When the tracking dye had reached the end of the gel power was switched

off. Six marker proteins (Sigma) of molecular weights 14.2K, 20.1K, 29K, 36K, 45K and 66K were run as controls.

4.2 Staining of gel

4.2.1 Coomassie blue stain (Weber and Osborn, 1969)

Gels were carefully removed from the plates and immersed in fixing solutions containing Coomassie blue stain in acetic acid, for 30 min. Gels were destained by several changes of destaining solutions. Gels were washed several times in distilled water for 30 min, then in 0.03% Na_2CO_3 for 10 min before drying under vacuum in a Biorad Model 483 slab dryer. Molecular weights of unknown bands could be estimated by interpolation on the scale generated by the marker proteins.

4.2.2 Silver staining (Oakley et al., 1980)

Silver staining was used to detect protein and glycoprotein components of EPM. Gels were handled wearing plastic gloves, rinsed with water to avoid contamination of the gels with protein secreted on the surface of the skin, and were agitated at all stages of the staining procedure. Gels were prefixed in 10% acetic acid, 30% methanol for 30 min. Glutaraldehyde (10%) was used as a fixative for 30 min. After fixing, gels were rinsed in a large volume of distilled water overnight. Gels were then given a final rinse in distilled water for

20 min. and placed in dithiothreitol (5 μg ml^{-1}) for 30 min. This solution was then poured off and 0.1% silver nitrate, freshly made up, was added without rinsing and shaken for a further 30 min. Gels were rinsed rapidly in distilled water and then twice in developer, which contained 37% formaldehyde (50 μl) in 100 ml of 3% Na_2CO_3, before being left to soak in a third volume of developer until the desired level of staining had been reached. Development was stopped by adding 2.3 M citric acid (5 ml) and mixing for 10 min.

5 DETERMINATION OF BLOOD GROUP AND SECRETOR STATUS

ABO and Lewis blood-group was determined for healthy buccal cell donors for whom secretor status was also established. Subjects were excluded if they had recently taken antibiotics. Blood was collected using an Autolet Lance to puncture the thumb tip of the donor. The red blood cells were then washed twice in saline and blood group was determined by agglutination, using standard laboratory procedures.

5.1 ABO blood group

The red blood cell suspension was adjusted to approximately 4% v/v and two separate drops were added to two glass tubes. One drop of either group A or group B serum kindly supplied by Mr J. Keary (Blood Bank, Glasgow Royal Infirmary) was added to each drop of red blood

cells. The tubes were observed macroscopically after 5 min for agglutination. Suitable control experiments were performed in parallel to the samples, where sterile saline was added instead of serum.

5.2 Lewis blood group

To 40 μl of either anti-Lewis a or anti-Lewis b serum in a glass test-tube was added 80 μl of a 3% (v/v) suspension of washed red cells in PBS. This was mixed thoroughly and incubated at room temperature for 15 min. The tubes were then centrifuged at 1000 g for 15 sec. The cell deposit was gently resuspended and observed macroscopically for agglutination.

5.3 Secretor status

To study secretor status the method described by Burford-Mason *et al*. (1988) was used. Saliva from each subject (2 ml) was collected in glass test-tubes, boiled for 20 min and then centrifuged at 5000 g for 10 min. The supernatant was either stored frozen at -20°C until tested, or used immediately. Secretor status was determined by a technique involving inhibition of agglutination. Saliva (40 μl) was incubated in round-bottomed test-tubes at room temperature for 45 min with an equal volume of either anti-A or anti-B monoclonal serum (kindly supplied by Mr J Keary, Blood Bank, Glasgow Royal Infirmary) or anti-H lectin (*Ulex*

europaeus; Inverclyde Biologicals Ltd.). The antisera were diluted in PBS before use so that they gave positive agglutination when tested against a panel of appropriate red cells. (The dilutions were 1:64 for both anti-A and anti-B and 1:2 for the anti-H). One drop of either A, B or O red cells screened for "private" antigens i.e. A, B or O (Glasgow Royal Infirmary, Department of Haematology) and diluted to 3% (v/v) in PBS was added to the tubes. The tubes were shaken and then incubated at room temperature for a further 30 min. Finally the tubes were centrifuged at 1000 g for 2 min and samples spread on glass slides. Positive agglutination was clearly identifiable, the red cells remaining in a pellet. Negative agglutination was taken as an indicator that the relevant blood group antigen was present in the test saliva. Saline controls were included on each plate.

6 F.P.L.C.

Fast high pressure liquid chromatography was kindly performed by Dr J.G. Lindsay (Department of Biochemistry, University of Glasgow) using a Pharmacia P.E.P. HR5/5 column. A sample of EPM (100 mg) was digested with N-glycanase, papain and mild alkali (see Sections 3.3.3.1 and 2) and adsorbed on an H-2 Synsorb matrix (see Section 3.3.4). Bound eluate (protein, 10 mg ml^{-1}; 100 μl) was run through the column at a flow rate of 40 ml h^{-1} using

trifluoroacetic acid buffer and 0 - 100 mM acetonitrile gradient. The sample was detected using ultraviolet spectrophometry (A_{280}).

7 STATISTICS

Student's t test was used to detect significant differences between adherence values and the results are presented as p-values. Standard errors of the mean are quoted for all adherence assay results.

8 CHEMICALS AND MEDIA

Sabouraud dextrose agar and yeast nitrogen base were from Difco. Acrylic was from Pharmacia. Sugars used in adherence studies were from Sigma. Glucose, sucrose and galactose used in growth media were from BDH Chemicals Ltd. Papain was from Sigma. N-Glycanase was from Genenzyme Fine Chemicals, Haverhill, Suffolk. Synsorb was from Chembiomed, Edmonton, Canada.

R E S U L T S

1 ADHESION OF *CANDIDA ALBICANS* AND OTHER *CANDIDA* SPECIES TO EPITHELIAL CELLS

1.1 Effect of time of incubation on adhesion of *Candida albicans* to buccal epithelial cells

The adhesion of *C. albicans* GDH 2346 to buccal epithelial cells with respect to time of incubation was investigated. Yeasts were grown to stationary phase in yeast nitrogen base medium containing 500 mM galactose. In all assays, the yeast concentration was 1×10^7 organisms ml^{-1}, that of the buccal cells was 1×10^5 cells ml^{-1}, and incubation periods of up to 90 min. were used. Figure 5 shows that adhesion reached a plateau after 45 min. with little increase in attachment after this time. In all subsequent assays, therefore, a 45 min. incubation period was used.

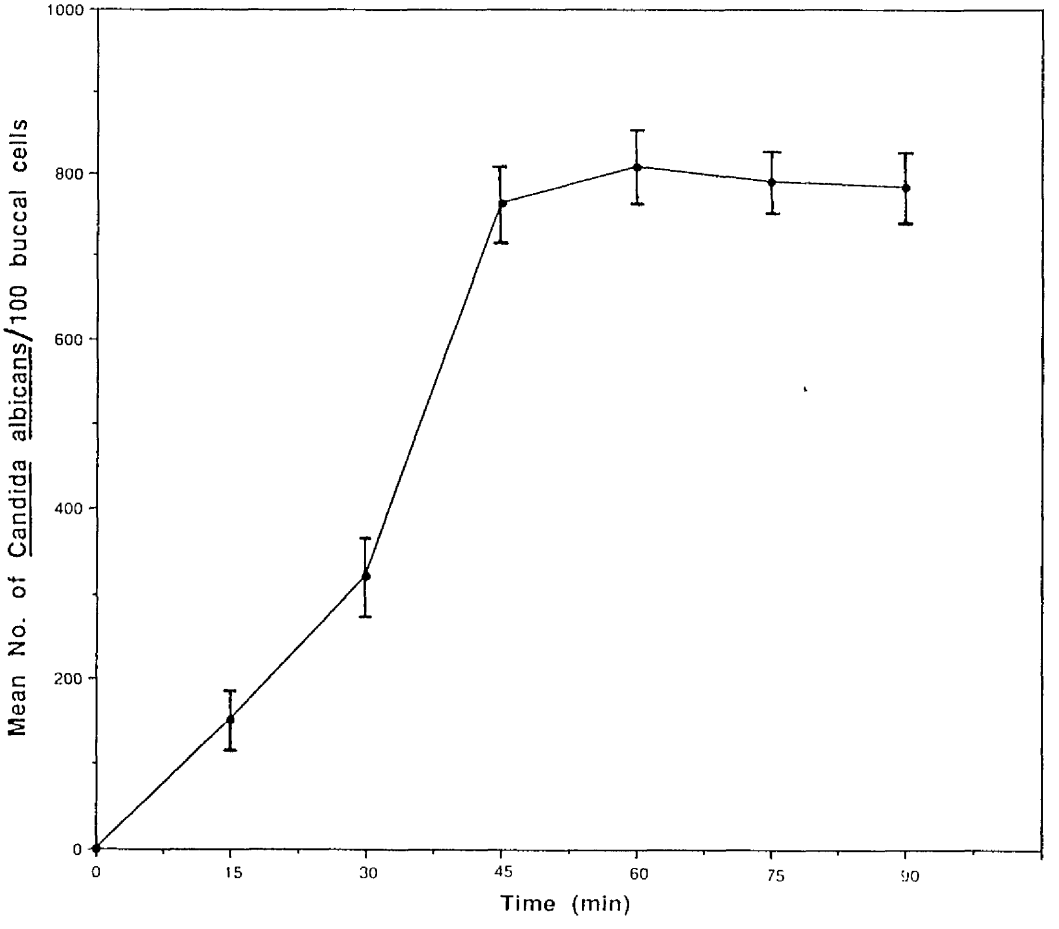
1.2 Adhesion of *Candida* isolates from leukoplakia patients to buccal epithelial cells

Oral leukoplakias, particularly non-homogeneous types, are often invaded by yeasts. Taxonomic investigations have shown that *Candida albicans* is by far the dominant species in leukoplakia lesions, constituting 82% of all yeasts identified (Krogh, 1989). Less frequently occurring biotypes of *C. albicans* are often found in advanced precancerous leukoplakias, suggesting a causal role for these biotypes in the malignant

Figure 5 Effect of time of incubation on adhesion of C. albicans GDH 2346 to buccal epithelial cells in vitro.

Bars indicate standard error of the mean.

The buccal cell donor was of blood group A, and a non-secretor (F.D.T.).


Mean No. of Candida albicans/100 buccal cells
1000
800
600
400
200
0
0
15
30
45
60
75
90
Time (min)

transformation. In this part of the investigation the adhesive ability of a number of leukoplakia isolates was assessed.

Ten Candida isolates from a variety of leukoplakia patients attending the outpatients clinic at the Royal Dental College, Copenhagen, Denmark were tested in assays with buccal epithelial cells. Details of the sites of isolation of these organisms are given in Table 3. For comparison, the well characterized denture stomatitis isolate C. albicans GDH 2346 was also included in this study. Initially, all the leukoplakia isolates were assumed to be C. albicans. However, isolates 909, 912, 936 and 937 failed to produce germ-tubes in serum after 4 h at 37°C (Table 8). These organisms were subsequently identified as C. glabrata, C. parapsilosis, C. lusitaniae and C. lusitaniae respectively by means of carbohydrate assimilation assays at the Medical Mycology Unit, University of Glasgow.

All isolates were tested for their ability to adhere to buccal epithelial cells, following growth to stationary phase in medium containing 500 mM galactose or 50 mM glucose, as infective strains have previously been shown to be up to ten times more adhesive after growth in high-galactose medium (McCourtie and Douglas, 1984). Only C. albicans 904 adhered to buccal cells in comparable numbers to the control strain, C. albicans GDH 2346 (Table 9). However isolates 902, 905, 906, 910 and

Table 8 Germ-tube production and sugar utilization by *Candida* isolates from leukoplakia patients

Species and isolate number		Germ-tube[1] production	Growth in yeast nitrogen base medium containing 50 mM Glucose	500 mM Sucrose	500 mM Galactose
C. albicans	902	+	+	+	+
	904	+	+	+	+
	905	+	+	+	+
	906	+	+	+	+
	910	+	+	+	+
	911	+	+	+	+
C. glabrata	909	-	+	+	-
C. parapsilosis	912	-	+	+	+
C. lusitaniae	936	-	+	+	+
C. lusitaniae	937	-	+	+	+
C. albicans	GDH2346	+	+	+	+

[1] Germ-tube production was assayed at hourly intervals for 4 h.

Table 9 Adhesion of different Candida isolates to human buccal epithelial cells after growth to stationary phase in yeast nitrogen base containing glucose, galactose or sucrose

Strain number	Carbon source[1]	Mean no. of adherent yeasts/100 epithelial cells ± SE	Relative Adhesion[2]	p[3]
C. albicans GDH 2346	Glucose	70 ± 10	1.0	
	Galactose	670 ± 10	9.6	<0.001
902	Glucose	49 ± 10	1.0	
	Galactose	320 ± 26	6.5	<0.001
904	Glucose	72 ± 18	1.0	
	Galactose	666 ± 62	9.3	<0.001
905	Glucose	4 ± 2	1.0	
	Galactose	271 ± 18	67.8	<0.001
906	Glucose	65 ± 9	1.0	
	Galactose	444 ± 44	6.8	<0.001
910	Glucose	55 ± 11	1.0	
	Galactose	347 ± 47	6.3	<0.001
911	Glucose	31 ± 3	1.0	
	Galactose	442 ± 35	14.3	<0.001
C. glabrata 909	Glucose	213 ± 68	1.0	
	Sucrose	126 ± 34	0.6	NS
C. parapsilosis 912	Glucose	94 ± 27	1.0	
	Galactose	31 ± 8	0.3	<0.01
C. lusitaniae 936	Glucose	57 ± 10	1.0	
	Galactose	136 ± 16	2.4	<0.001
C. lusitaniae 937	Glucose	118 ± 14	1.0	
	Galactose	119 ± 22	1.0	NS

1 The carbon source was either glucose (50 mM), galactose (500 mM) or sucrose (500 mM) as indicated.

2 Adhesion is calculated relative to that of glucose-grown cells.

3 Probability values comparing adhesion of galactose- or sucrose-grown yeasts of the same strain; NS, not significant.

Results represent mean ± standard error of two independent determinations performed in triplicate.

The buccal cell donor was blood group A, and a non-secretor (F.D.T.).

911 were able to adhere in significantly higher numbers after growth on 500 mM galactose than after growth in 50 mM glucose. Isolate 905 gave the highest relative adhesion value, but this was due to the poor ability of glucose-grown yeasts of this strain to adhere to buccal cells. Even when very high numbers of yeasts were used (e.g. 1×10^9 ml^{-1}) adhesion was poor. Isolate 911 also produced a high relative adhesion value of 14.3. In contrast, isolates 902, 906 and 910 had relative adhesion values of 6.5, 6.8 and 6.3 respectively. Previous work has shown that infective (I) strains of C. albicans modify their cell surface when grown in high concentrations of galactose, whereas carrier (C) strains do not (McCourtie and Douglas, 1984). Although very little is known about the exact origin of the leukoplakia isolates, these results indicate that all of the C. albicans strains show an ability to modify their cell surfaces in response to galactose.

Adhesion of several Candida species to buccal cells has been described before (Critchley and Douglas, 1985), but this is the first report of assays with C. lusitaniae and C. glabrata. One of the two isolates of C. lusitaniae showed no significant enhancement of adhesion when grown in high concentrations of galactose. Similarly, C. glabrata 909 failed to show increased adhesion after growth in high-sucrose medium. On the other hand, C. parapsilosis 912 was significantly less

adherent after growth on galactose. However, this organism grew rather poorly in high-galactose medium; routinely inocula were incubated for 24 h prior to subculture for a further 24 h.

2 CRUDE EPM

2.1 Effect of crude EPM on adhesion of C. albicans

Adhesion of C. albicans to epithelial cells is thought to involve lectin-like interactions between yeast adhesins and host-cell receptors. Mannoprotein isolated from culture supernatants of galactose-grown organisms blocks adhesion to buccal or vaginal cells in vitro. This extracellular material originates, at least in part, from a fibrillar surface layer and its ability to inhibit adhesion indicates that it contains yeast adhesin. Throughout this study, crude EPM was isolated by means of ultrafiltration of culture supernatants, followed by a short (48 h) period of dialysis. With this method, yields of EPM (15-20%) were similar to those obtained by acetone precipitation (McCourtie and Douglas, 1985) or by prolonged dialysis (Critchley and Douglas, 1987a). EPM was produced more rapidly than by the dialysis-only method, and was equally effective in inhibiting adhesion of C. albicans GDH 2346 to buccal epithelial cells. The data shown in Table 10 indicate that crude EPM preparations obtained by either dialysis method were similar in protein content (6.15 and 6.53%) and in their

Table 10 Effect of crude EPM from C. albicans GDH 2346 on adhesion of strain GDH 2346 to buccal epithelial cells[1]

Method of preparation of EPM	Adherence relative to control[2]	Protein content of EPM (% dry weight)	P[3]
Dialysis only	0.51	6.15	<0.001
Ultrafiltration followed by dialysis	0.50	6.53	<0.001
Control (no EPM)	1.00		

[1] Epithelial cells were preincubated with 10 mg ml^{-1} at 37°C for 30 minutes, then used in adhesion assays with a yeast concentration of 1 x 10^7 organisms ml^{-1}.

[2] Adherence is expressed relative to that of a control (PBS-treated buccal cells) for which a value of 896 ± 54 adherent yeasts per 100 epithelial cells (mean ± SEM) was obtained.

[3] Probability values relative to the control.

Results represent mean of three independent assays performed in triplicate.

The buccal cell donor was blood group A, and a non-secretor (F.D.T.).

ability to inhibit adhesion (50-51%).

2.1.1 Biochemical analysis of crude EPM from five different strains

EPM preparations were analysed for carbohydrate, protein and phosphorus. The results are shown in Table 11. Carbohydrate was the major component, accounting for over 72% of the dry weight. The protein content ranged from 7.2% (C. albicans GDH 2346) to 10.5% (C. albicans GDH 2023); there were however small batch-to- batch variations as seen with the crude EPM used to perform the experiment described in Table 10. The phosphorus content ranged from 0.41% in C. albicans GDH 2346 to 0.73% in C. albicans MRL 3153. These analytical results are similar to those described by McCourtie and Douglas (1985) and Critchley and Douglas (1987a).

SDS PAGE of crude EPM preparations was carried out with 15% separating gels. Plate 1 shows a typical result obtained with EPM from the C. albicans strains indicated. Proteins visualized by the silver staining technique varied in molecular mass from 13 kDa to 200 kDa. Two of the samples run on the gel were from batches of crude EPM prepared by prolonged dialysis of C. albicans culture supernatant and stored in the freeze-dried state for three years; the other six samples were freshly prepared using the ultrafiltration technique detailed in Materials and Methods 3.3.1.1. There were no major discernible differences in the staining profiles of the samples.

Table 11 Biochemical analysis of EPM isolated from culture supernates of C. albicans

Strain No.	Carbohydrate content (% ± SEM)	Protein content (% ± SEM)	Phosphorus content (% ± SEM)
GDH 2346	79.2 ± 1.7	7.2 ± 0.3	0.41 ± 0.09
GDH 2023	72.4 ± 0.8	10.5 ± 0.3	0.47 ± 0.04
MRL 3153	81.3 ± 1.4	7.6 ± 0.1	0.73 ± 0.14
GRI 681	77.0 ± 1.8	9.7 ± 0.4	0.45 ± 0.01
GRI 682	75.2 ± 1.7	8.6 ± 0.4	0.44 ± 0.01

Results represent means of two independent determinations performed in triplicate.

Plate 1 SDS PAGE gel of eight crude EPM samples run on a 5% stacking and 15% separating gel

[1] EPM samples prepared by Critchley (1986)

kD
Standards
C. albicans GDH 2346
C. albicans GDH 2023
C. albicans MRL 3153
C. albicans GRI 681
C. albicans GRI 682
C. albicans 'Outbreak'
C. albicans GDH 2346 1
C. albicans GDH 2023 1
66 >
45 >
36 >
29 >
20.1 >
14.2 >

2.2 "Lectin-like" material isolated from crude EPM by affinity chromatography

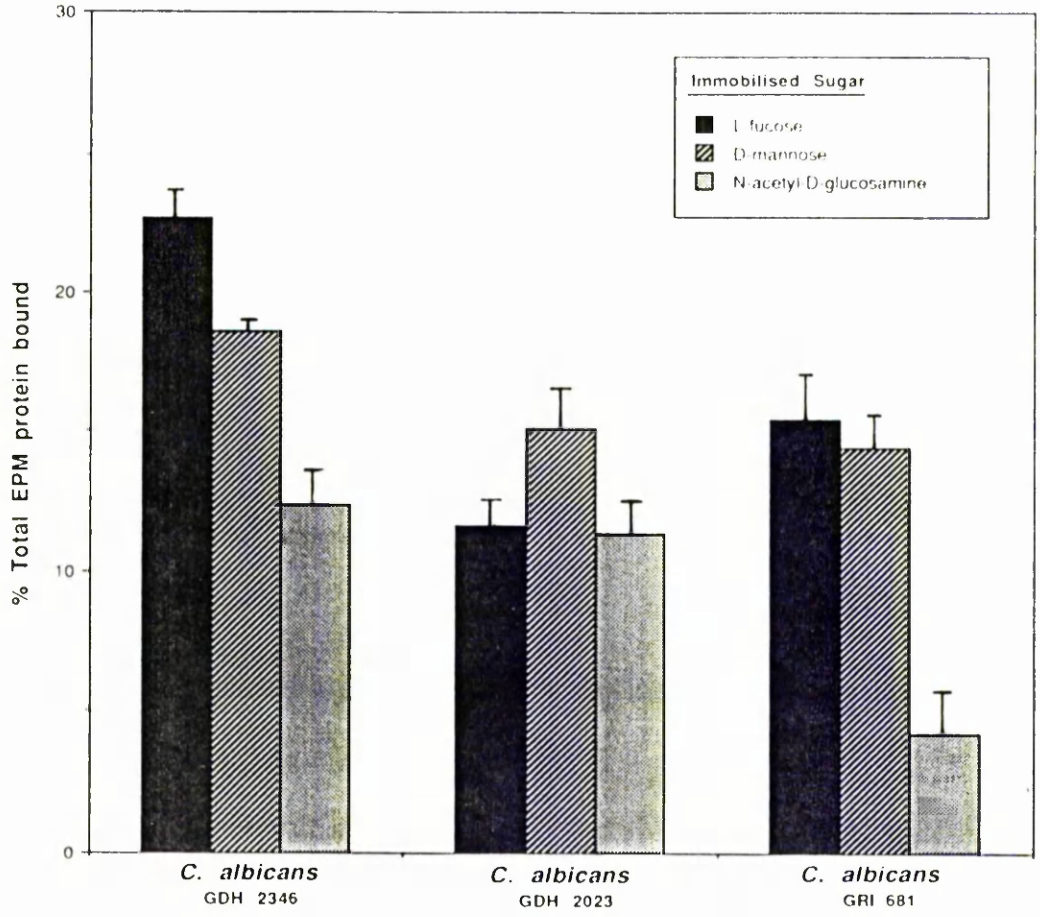
To investigate the presence of lectin-like adhesins in C. albicans, crude EPM preparations from three strains - GDH 2346, GDH 2023 and GRI 681 - were applied to affinity columns which contained three different sugars immobilized on epoxy-activated Sepharose. The proportion of EPM protein bound to each column was then determined. Figure 6 shows that all three preparations contained proportions of material with lectin-like binding specificities for L-fucose, D-mannose and N-acetyl-D-glucosamine. With C. albicans GDH 2346, for example, 22.6% of the total EPM protein bound to immobilized L-fucose, whilst 18.0% and 12.3% bound to D-mannose and N-acetyl-D-glucosamine respectively. Similarly, although rather less EPM protein from strain GRI 681 bound to affinity columns, fucose-binding protein was the most abundant and N-acetyl-D-glucosamine the least abundant type. By contrast, with EPM isolated from C. albicans GDH 2023, most of the bound protein was associated with D-mannose, whilst approximately equal amounts bound to L-fucose and N-acetyl-D-glucosamine.

2.2.1 Effect of different "lectin-like" components from EPM on adhesion of C. albicans

Bound protein, eluted from epoxy-activated Sepharose 6B columns by means of the appropriate sugar, was

Figure 6 Percentage of total EPM bound (±SEM) to three different immobilized sugars

Bars indicate standard error of the mean.


30
20
10
0
% Total EPM protein bound
Immobilised Sugar
L-fucose
D-mannose
N-acetyl-D-glucosamine
C. albicans
GDH 2346
C. albicans
GDH 2023
C. albicans
GRI 681

concentrated and applied to a Sepharose G-25 desalting column. An A_{280} profile of the eluate was determined, and fractions constituting the protein peak were pooled. Each fraction was then tested for its ability to inhibit adhesion of homologous yeasts (Tables 12 and 13) and the Adherence Inhibition Index (AII; see Appendix III) calculated (Tables 14 and 15).

When purified material from C. albicans GDH 2346 was used in adhesion inhibition experiments with strain GDH 2346, L-fucose-bound protein and N-acetyl-D-glucosamine-bound protein were more effective inhibitors (on a protein weight basis) than protein bound to the D-mannose column (Table 14). By contrast, with C. albicans GDH 2023 and homologous protein, D-mannose-specific protein was the best inhibitor, while L-fucose- and N-acetyl-D-glucosamine-bound protein were slightly less effective (Table 15). If these results are compared with Critchley (1986) it is apparent that there is a marked difference in the behaviour of the buccal cells from different individuals.

The AII values calculated are shown in Tables 14 and 15. The differing lectins have different effects on the two strains. These effects had not been noted previously and were unexpected. The protocol was therefore repeated, and the results were found to be reproducible with respect to the individual buccal cell donor.

Table 12 Effect of different lectin-like components from crude EPM from C. albicans GDH 2346 on the adhesion of a homologous yeast strain

Pretreatment of buccal cells		Mean No. of yeasts per 100 BEC's ± SEM	Percentage inhibition
None (PBS control)	(1)	951 ± 58	0
	(2)	506 ± 23	0
EPM (10 mg ml^{-1})	(1)	424 ± 38	55.4
from strain GDH 2346	(2)	261 ± 13	48.4
L-fucose-bound	(1)	404 ± 37	57.4
protein	(2)	275 ± 19	45.6
D-mannose-bound	(1)	687 ± 94	27.8
protein	(2)	468 ± 19	7.5
N-acetyl-D-glucosamine-	(1)	422 ± 31	55.6
bound protein	(2)	281 ± 17	44.5

1, 2 refer to two separate experiments, the means of which are shown, and are from three independent determinations performed in triplicate.

Adhesion assays were performed using 1 x 10^7 yeasts ml^{-1}. The buccal cell donor was blood group A, and a non-secretor (F.D.T.).

Table 13 Effect of different lectin-like components from crude EPM from C. albicans GDH 2023 on the adhesion of a homologous yeast strain

Pretreatment of buccal cells	Mean No. of yeasts per 100 BEC's ± SEM	Percentage inhibition
None (PBS control)	(1) 2383 ± 107	0
	(2) 1283 ± 43	0
EPM (10 mg ml^{-1})	(1) 1125 ± 76	52.8
from strain GDH 2023	(2) 669 ± 34	47.8
L-fucose-bound	(1) 1286 ± 68	46.0
protein	(2) 738 ± 20	42.5
D-mannose-bound	(1) 1139 ± 67	52.2
protein	(2) 881 ± 20	31.3
N-acetyl-D-glucosamine-	(1) 1222 ± 82	48.7
bound protein	(2) 764 ± 38	40.5

1, 2 refer to two separate experiments, the means of which are shown, and are from three independent determinations performed in triplicate.

Adhesion assays were performed using 1 x 10^7 yeasts ml^{-1}.

The buccal cell donor was blood group A, and a non-secretor (F.D.T.).

Table 14 Adhesion Inhibition Index values for lectin-like material isolated from the EPM of C. albicans GDH 2346 by affinity chromatography

Sugar coupled to Sepharose gel	Adhesion Inhibition Index 1st Assay	2nd Assay
L-fucose	30.5	28.7
D-mannose	8.7	11.3
N-acetyl-D-glucosamine	31.1	24.1

Table 15 Adhesion Inhibition Index values for lectin-like material isolated from the EPM of C. albicans GDH 2023 by affinity chromatography

Sugar coupled to Sepharose gel	Adhesion Inhibition Index 1st Assay	2nd Assay
L-fucose	19.2	17.2
D-mannose	22.7	21.2
N-acetyl-D-glucosamine	16.1	11.7

2.2.2 Effect of different sugars on the adherence of C. albicans to buccal epithelial cells

With C. albicans GDH 2023, the sugars L-fucose, D-mannose and N-acetyl-D-glucosamine, when incorporated in adhesion assays at 25 mg ml^{-1}, possessed the ability to inhibit adherence to buccal cells by 39%, 43% and 35% respectively (Table 16). On the other hand, with C. albicans GDH 2346, which had previously shown a different pattern of adhesion (Tables 14 and 15), the dual effect of L-fucose and N-acetyl-D-glucosamine was tested; the inhibition of adherence was significantly ($P < 0.001$) increased relative to the effect of the two sugars when assayed independently, with adherence being only 69% relative to the control (Table 17).

2.2.3 Effect of C. albicans "lectin-like" proteins on the adhesion of heterologous strains

Possible inhibition by the different lectin-like EPM components on heterologous strains of C. albicans was investigated. Partially purified fractions of EPM from C. albicans GDH 2023 had no significant effect on the adhesion of C. albicans GDH 2346 (Table 18). This was in agreement with previous results (McCourtie and Douglas, 1985; Critchley and Douglas, 1987b) which showed that crude EPM from C. albicans GDH 2023 had no effect on the adhesion of strain GDH 2346, thus indicating the specificity of yeast adhesin. In contrast L-fucose and N-acetyl-D-glucosamine-specific lectins isolated from

Table 16 Effect of different sugars on adhesion of C. albicans GDH 2023 to buccal epithelial cells

Sugar present in assay mixture	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	p[2]
None (PBS control)	1088 ± 22	100	-
L-fucose	699 ± 25	61	<0.001
D-mannose	623 ± 37	57	<0.001
N-acetyl-D-glucosamine	709 ± 62	65	<0.001

[1] Adherence relative to that of yeasts suspended in PBS.

[2] Probability values relative to adhesion of PBS control.

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using 1×10^7 yeasts ml^{-1}.

The buccal cell donor was blood group A, and a non-secretor (F.D.T.)

Table 17 Effect of different sugars on adhesion of C. albicans GDH 2346 to buccal epithelial cells

Sugar present in assay mixture	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	p[2]
None (PBS control)	480 ± 28	100	-
L-fucose	385 ± 22	80	<0.001
N-acetyl-D-glucosamine	382 ± 45	80	<0.001
L-fucose/N-acetyl-D-glucosamine	333 ± 29	69	<0.001

[1] Adherence relative to that of yeasts suspended in PBS.

[2] Probability values relative to adhesion of PBS control.

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using 1×10^7 yeasts ml^{-1}.

The buccal cell donor was blood group A, and a non-secretor (F.D.T.)

Table 18 Effect of EPM protein from C. albicans GDH 2023 on the adhesion of C. albicans GDH 2346 to buccal cells

Pretreatment of buccal cells	Mean no. of yeasts per 100 BEC's ± SEM	Percentage Inhibition (and significance[1])	Adhesion[2] Inhibition Index
None (PBS control)	472 ± 12	0	-
EPM (10 mg ml^{-1} from C. albicans GDH 2023)	471 ± 17	0 (NS)	0
L-fucose-bound protein	445 ± 21	5.7(NS)	2.3
D-mannose-bound protein	448 ± 13	5.1(NS)	3.4
N-acetyl-D-glucosamine-bound protein	437 ± 12	7.4(NS)	2.1

[1] NS, not significant;

[2] Adhesion inhibition index for protein relative to effect of crude EPM from C. albicans GDH 2346 on the adhesion of strain GDH 2346 to buccal cells.

Results represent means of three independent determinations performed in triplicate.

The buccal cell donor was blood group A, and a non-secretor (F.D.T.)

C. albicans GDH 2346 inhibited adhesion of C. albicans GDH 2023, whereas the D-mannose-specific lectin had no effect (Table 19).

3 PURIFICATION OF THE YEAST ADHESIN

3.1 Effect of chemical and enzymic pretreatments on the ability of crude EPM to inhibit adhesion of C. albicans GDH 2346 to buccal epithelial cells

3.1.1 Pretreatment with mild alkali

Mild alkali has previously been reported to enhance the ability of EPM to inhibit adhesion of C. albicans GDH 2346 to buccal epithelial cells (Critchley and Douglas, 1987b). However the optimal conditions for pretreatment have never been investigated. Mild alkali has been shown to eliminate from yeast mannoprotein the base-labile oligosaccharides linked through serine and threonine residues to the protein part of the molecule (Ballou, 1976). In the present study, the effect of pretreatments with different concentrations of NaOH on the ability of crude EPM to inhibit adherence of C. albicans GDH 2346 to buccal epithelial cells was compared with that of pretreatment with PBS.

The results (Figure 7) indicate that pretreatment with NaOH at a concentration of 0.1 M (the concentration previously used by Critchley and Douglas, 1987b) produces maximal inhibition of adhesion. This concentration was

Table 19 Effect of EPM protein from C. albicans GDH 2346 on the adhesion of C. albicans GDH 2023 to buccal cells

Pretreatment of buccal cells	Mean no. of yeasts per 100 BEC's ± SEM	Percentage Inhibition (and significance[1])	Adhesion[2] Inhibition Index
None (PBS control)	1236 ± 60	0 (NS)	-
EPM (10 mg ml^{-1} from C. albicans GDH 2346)	904 ± 18	27 (*)	-
L-fucose-bound protein	849 ± 68	31 (*)	20.1
D-mannose-bound protein	1295 ± 25	0 (NS)	0
N-acetyl-D-glucosamine-bound protein	901 ± 33	27 (*)	15.1

[1] NS, not significant; * $P < 0.001$

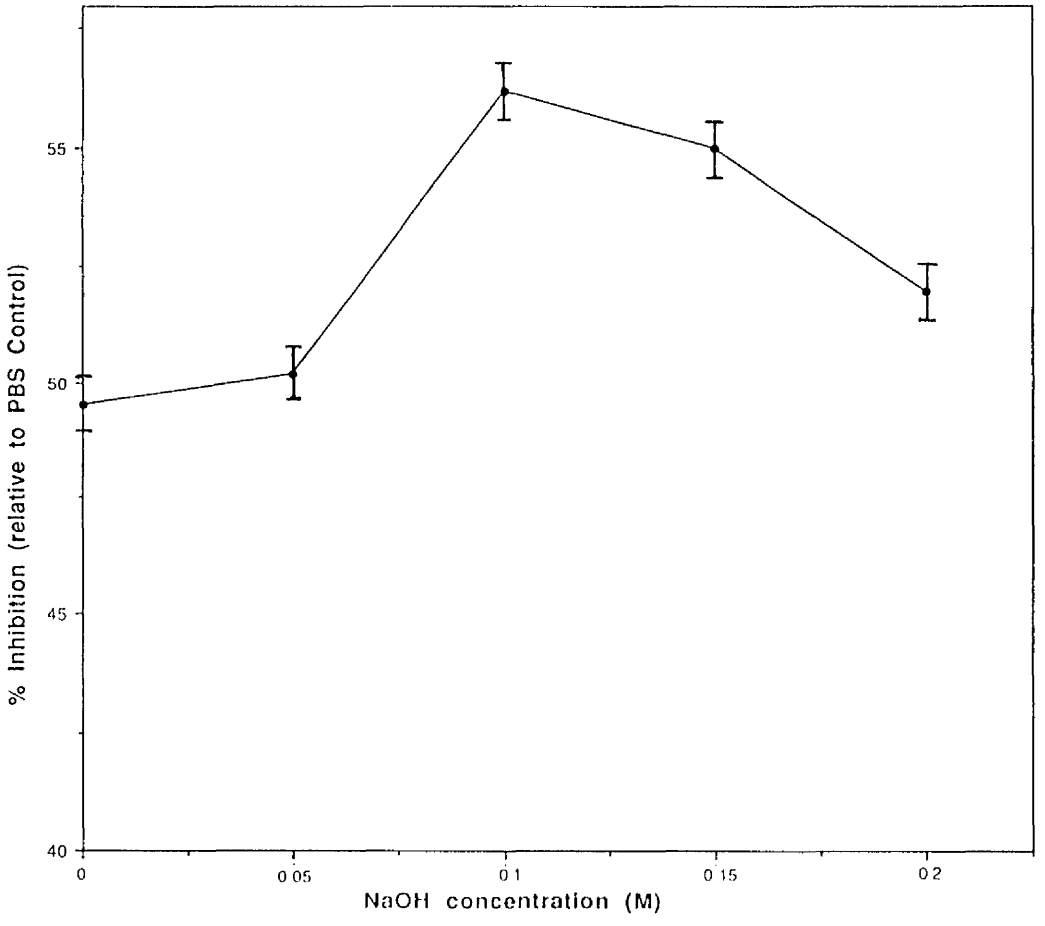
[2] Adhesion inhibition index for protein relative to effect of crude EPM from C. albicans GDH 2023 on the adhesion of strain GDH 2023 to buccal cells.

Results represent means of three independent determinations performed in triplicate.

The buccal cell donor was blood group A, and a non-secretor (F.D.T.)

Figure 7 Effect of pretreatment with different concentrations of NaOH on the ability of crude EPM to inhibit the adhesion of C. albicans GDH 2346 to buccal epithelial cells

Bars indicate standard error of the mean


% Inhibition (relative to PBS Control)
55
50
45
40
0
0 05
0 1
0 15
0 2
NaOH concentration (M)

used in all subsequent experiments. Higher alkali concentrations produced less inhibition and it is possible that there was some hydrolysis of the protein portion of the mannoprotein under these conditions.

3.1.2 Pretreatment with papain

Previous work (Critchley and Douglas, 1987b) has shown that partial degradation of EPM from C. albicans GDH 2346 by papain digestion, followed by mild alkali treatment, increases the effectiveness of EPM as an inhibitor of adhesion. This increased inhibition was attributed to the production of peptide fragments which bound more easily to epithelial cell receptors. In the present study the effects of combined papain/mild alkali pretreatment of EPM were further investigated. The results confirmed the increased inhibition described by Critchley and Douglas (1987b); this is shown in Table 20 which contains results from experiments with buccal cells from a secretor of blood group antigens. The effect is independent of secretor status as shown in Table 21, where the buccal cells were from a donor who was a non-secretor of blood group antigens.

Papain/mild alkali pretreatment of EPM from C. albicans GDH 2023 similarly enhanced the effectiveness of this material as an inhibitor (Table 22). Such pretreatment increased EPM inhibition of adhesion of the homologous yeast strain from 44% to 64%. Overall, these results with EPM preparations from two strains indicate

Table 20 Effect of papain/mild alkali on the ability of EPM from C. albicans GDH 2346 to inhibit adherence of the homologous yeast strain to buccal epithelial cells from a secretor

Pretreatment of EPM	Mean no. of adherent yeasts/100 epithelial cells ± SEM	Percentage inhibition of adherence[1]	p[2]
Control (No EPM)	768 ± 16	0	-
None (Crude EPM)	377 ± 22	51	<0.001
Mild alkali	357 ± 18	56	<0.001
Papain; mild alkali	229 ± 9	74	<0.001

[1] Inhibition relative to PBS control.

[2] Probability values relative to the control.

Buccal epithelial cells were incubated at 37°C for 30 min. in a solution (10 mg ml^{-1}) of EPM pretreated as indicated. Control buccal cell preparations were incubated under the same conditions in PBS.

The buccal cell donor was blood group A, secretor (H.S.).

Table 21 Effect of papain/mild alkali on the ability of EPM from C. albicans GDH 2346 to inhibit adherence of the homologous yeast strain to buccal epithelial cells from a non-secretor

Pretreatment of EPM	Mean no. of adherent yeasts/100 epithelial cells ± SEM	Percentage inhibition of adherence[1]	p[2]
Control (No EPM)	849 ± 48	0	-
None (Crude EPM)	463 ± 22	47	<0.001
Papain: mild alkali	275 ± 17	69	<0.001

[1] Inhibition relative to PBS control.

[2] Probability values relative to the control.

Buccal epithelial cells were incubated at 37°C for 30 min. in a solution (10 mg ml^{-1}) of EPM pretreated as indicated. Control buccal cell preparations were incubated under the same conditions in PBS.

The buccal cell donor was blood group A, non-secretor (F.D.T.).

Table 22 Effect of papain/mild alkali on the ability of EPM from C. albicans GDH 2023 to inhibit adherence of the homologous yeast strain to buccal epithelial cells from a non-secretor

Pretreatment of EPM	Mean no. of adherent yeasts/100 epithelial cells ± SEM	Percentage inhibition of adherence[1]	p[2]
Control (No EPM)	1502 ± 86	0	-
None (Crude EPM)	848 ± 41	44	<0.001
Papain: mild alkali	684 ± 41	64	<0.001

[1] Inhibition relative to PBS control.

[2] Probability values relative to the control.

Buccal epithelial cells were incubated at 37°C for 30 min. in a solution (10 mg ml^{-1}) of EPM pretreated as indicated. Control buccal cell preparations were incubated under the same conditions in PBS.

The buccal cell donor was blood group A, non-secretor (F.D.T.).

that this degradation protocol produces fragments that are more effective inhibitors of adhesion. The specificity of the treatments involved was confirmed in suitable control experiments (Table 23).

3.2 Affinity adsorption of EPM protein from C. albicans

3.2.1 Affinity adsorption of EPM protein using epoxyactivated Sepharose

A simple protocol was developed using epoxyactivated Sepharose 6B-beads. EPM solution was incubated with Sepharose, to which had been bound either L-fucose, D-mannose or N-acetyl-D-glucosamine. Bound protein was eluted using the appropriate sugar. This batch technique for binding EPM protein gave a similar pattern of results (Figure 8) to those obtained earlier (Figure 6) where columns were used.

3.2.2 Binding of EPM protein and carbohydrate to Synsorb matrices

Following the observation that mixtures of glycosides appeared to be significantly better inhibitors of adhesion than single glycosides, immobilized oligosaccharides were used to further purify the yeast adhesin. Crude EPM was bound and eluted from four different blood-group oligosaccharides immobilized on a silica matrix (Synsorb). Biochemical analyses were performed on the eluates. EPM preparations from three

Table 23 Specificity of chemical and enzymic treatments on the ability of crude EPM to inhibit adhesion of C. albicans GDH 2346 to buccal epithelial cells

Pretreatment of EPM	Mean no. of adherent yeasts/100 buccal cells ± SEM	Relative[4] Adhesion	p[5]
None	345 ± 10	50	<0.001
Mild alkali	306 ± 34	44	<0.001
Papain + mild alkali	243 ± 17	35	<0.001
Acid + alkali (t_0)[1]	351 ± 11	49	<0.001
Papain + $\alpha_2$macroglobulin (t_0)[2]	388 ± 25	56	<0.001
$\alpha_2$macroglobulin[3]	337 ± 29	51	<0.001
Control (No EPM)	693 ± 37	100	-

[1] Mild alkali (NaOH, 0.1 M) and mild acid (HCl, 0.1 M) were mixed prior to the addition of crude EPM (10 mg ml^{-1}).

[2] Papain and $\alpha_2$macroglobulin were mixed prior to the addition of crude EPM (10 mg ml^{-1}).

[3] $\alpha_2$macroglobulin was dissolved in PBS, prior to the addition of crude EPM (10 mg ml^{-1}).

Table 23 (continued)

[4] Adhesion is expressed as a percentage of that of control. Prior to adhesion assays, the buccal cells were incubated at 37°C for 30 min. in a solution (10 mg ml^{-1}) of crude EPM. Control buccal cell preparations were incubated under the same conditions in PBS. Adhesion assays were performed twice in triplicate, with a yeast cell concentration of 1×10^{7} ml^{-1}.

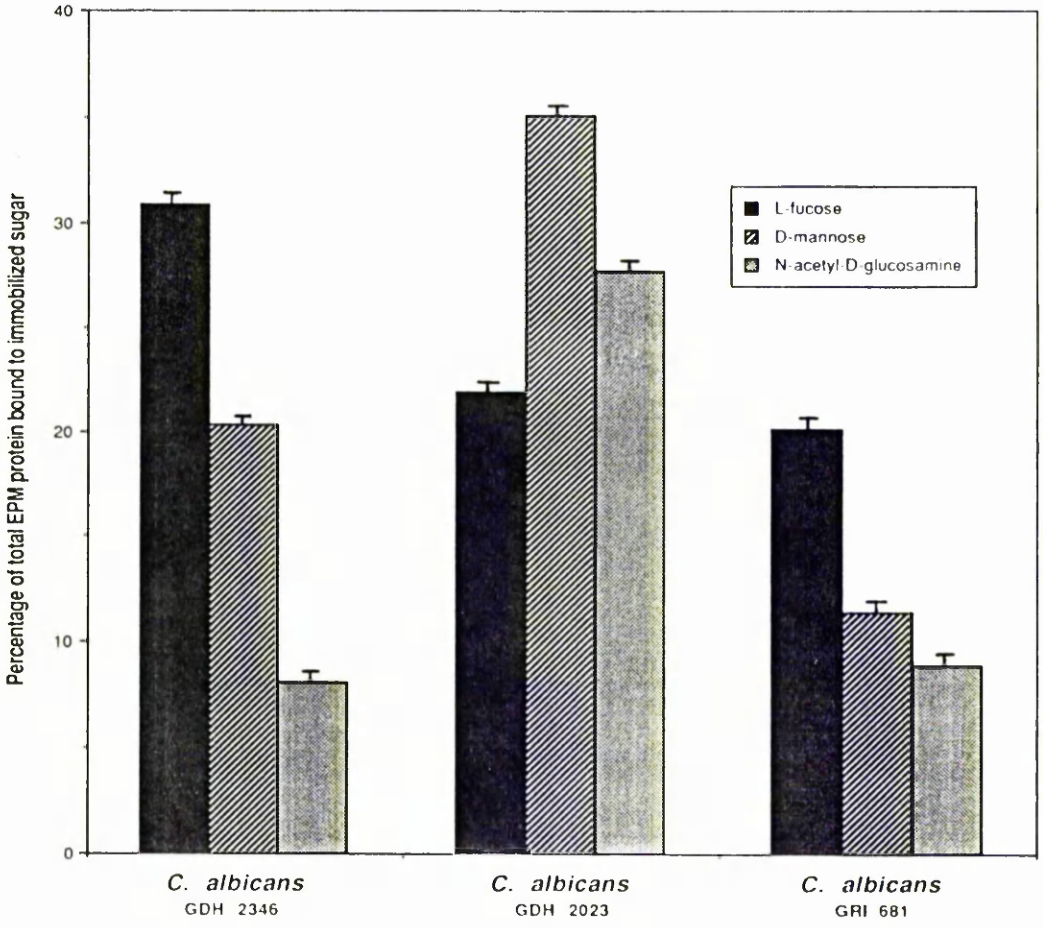
[5] Probability values relative to the adhesion of PBS control.

The buccal cell donor was of blood group A, non-secretor (F.D.T.).

The donors of the buccal cells in all of these experiments were of blood group A.

Figure 8 Percentage of total EPM protein bound to three different immobilized sugars as measured by batch affinity adsorption

Bars indicate standard error of the mean


Percentage of total EPM protein bound to immobilized sugar
40
30
20
10
0
L-fucose
D-mannose
N-acetyl-D-glucosamine
C. albicans
GDH 2346
C. albicans
GDH 2023
C. albicans
GRI 681

different strains of C. albicans were used.

The results (Tables 24, 25, 26) indicated that significant amounts of EPM bound to the oligosaccharides. EPM protein from C. albicans GDH 2346 bound in lowest amounts to the H-1 oligosaccharide (5.1%), whilst the binding to the Lewis-a, Lewis-b and H-2 oligosaccharides was 14.4%, 12.2% and 13.4% respectively (Table 24). With C. albicans GDH 2023 there was the greatest binding to the H-2 oligosaccharide (24.8%) and least to the Lewis-b oligosaccharide (4.5%) (Table 25). On the other hand, the EPM protein from strain GRI 681 showed some predilection for the Lewis-b oligosaccharide (Table 26). These results are summarized in Figure 9.

3.3 Efficiency of partially purified adhesin in the inhibition of yeast adhesion

3.3.1 The use of partially purified adhesin from C. albicans GDH 2346 to inhibit adhesion

To determine which of the bound fractions showed the greatest inhibition of adherence relative to crude EPM, a series of inhibition assays was performed; in each assay C. albicans GDH 2346 was used with a buccal cell donor of blood group A (a non-secretor). The results of these experiments are shown in Tables 27, 28, 29 and 30. The AII values are summarized in Table 31, from which it can be seen that H-2 bound material had the highest AII of 62.2, and Lewis-b bound material had the lowest AII of

Table 24 Biochemical analysis of EPM from C. albicans GDH 2346 bound to Synsorb

Immobilized Oligosaccharide (Trivial Name)	Percentage of crude EPM[1] (±SEM) bound as	
	Protein	Carbohydrate
βGal(1-3)βGlcNAc | (1-4) αFuc (Lewis a)	14.4±0.4	20.1±1.0
αFuc(1-2)βGal(1-3)βGlcNAc | (1-4) αFuc (Lewis b)	12.2±0.2	16.0±0.5
αFuc(1-2)βGal(1-3)βGlcNAc (H 1)	5.1±0.1	7.9±0.1
αFuc(1-2)βGal(1-4)βGlcNAc (H 2)	13.4±0.2	8.3±0.1

[1] Results represent means of two independent determinations performed in triplicate.

Samples of EPM (50 mg ml^{-1}; 0.2 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was then eluted with a 1.5% solution of NH_4OH in sterile saline.

Table 25 Biochemical analysis of EPM from C. albicans GDH 2023 bound to Synsorb

Immobilized Oligosaccharide (Trivial Name)	Percentage of crude EPM[1] (±SEM) bound as	
	Protein	Carbohydrate
βGal(1-3)βGlcNAc | (1-4) αFuc (Lewis a)	18.7±0.2	8.5±0.3
αFuc(1-2)βGal(1-3)βGlcNAc | (1-4) αFuc (Lewis b)	4.5±0.2	5.5±0.1
αFuc(1-2)βGal(1-3)βGlcNAc (H 1)	10.4±0.1	12.4±0.2
αFuc(1-2)βGal(1-4)βGlcNAc (H 2)	24.8±0.4	11.7±0.2

[1] Results represent means of two independent determinations performed in triplicate.

Samples of EPM (50 mg ml^{-1}; 0.2 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was then eluted with a 1.5% solution of NH_4OH in sterile saline.

Table 26 Biochemical analysis of EPM from C. albicans GRI 681 bound to Synsorb

Immobilized Oligosaccharide (Trivial Name)	Percentage of crude EPM[1] (±SEM) bound as	
	Protein	Carbohydrate
βGal(1-3)βGlcNAc | (1-4) αFuc (Lewis a)	8.6±0.2	18.4±0.4
αFuc(1-2)βGal(1-3)βGlcNAc | (1-4) αFuc (Lewis b)	13.7±0.2	27.4±0.6
αFuc(1-2)βGal(1-3)βGlcNAc (H 1)	6.1±0.1	13.0±0.2
αFuc(1-2)βGal(1-4)βGlcNAc (H 2)	8.5±0.1	12.6±0.3

[1] Results represent means of two independent determinations performed in triplicate.

Samples of EPM (50 mg ml^{-1}; 0.2 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was then eluted with a 1.5% solution of NH_4OH in sterile saline.

Figure 9 Percentage of total EPM protein bound to four different immobilized oligosaccharides

Oligosaccharide coupled to Synsorb	Trivial name
 βGal(1-3)βGlcNAc (1-4) αFuc	Lewis a
 αFuc(1-2)βGal(1-3)βGlcNAc (1-4) αFuc	Lewis b
 αFuc(1-2)βGal(1-3)βGlcNAc	H 1
 αFuc(1-2)βGal(1-4)βGlcNAc	H 2

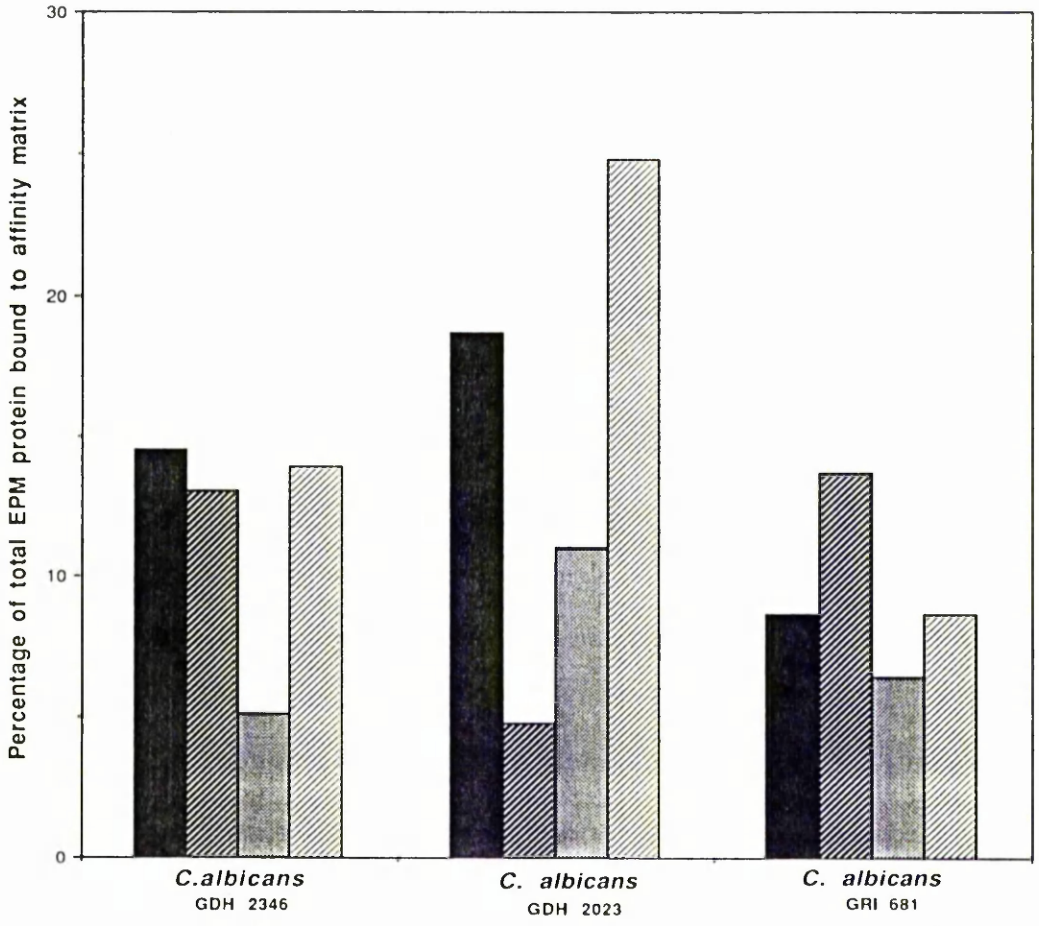

Percentage of total EPM protein bound to affinity matrix
30
20
10
0
C.albicans
GDH 2346
C. albicans
GDH 2023
C. albicans
GRI 681

Table 27 Synsorb Lewis-a for adhesin purification: inhibition of adhesion of C. albicans GDH 2346 to buccal epithelial cells by EPM fractions

Pretreatment of EPM	Mean no. of adherent yeasts/ 100 epithelial cells ± SEM	Percentage inhibition of adherence	AII[2]
Lewis-a bound[1]	372 ± 33	52	46.7
Lewis-a eluted[1]	608 ± 33	22	2.0
None	411 ± 22	48	1.0
PBS Control (No EPM)	783 ± 14	0	-

[1] Samples of EPM from strain GDH 2346 (100 mg ml^{-1}; 0.2 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. Both eluted and bound fractions were lyophilized and reconstituted in distilled water and desalted on a Sephadex G-25 desalting column (Pharmacia). Appropriate material was then incubated with buccal epithelial cells (37°C for 30 min) before their use in adhesion assays.

[2] Adhesion inhibition index (AII) calculated relative to untreated EPM. The buccal cell donor was blood group A and a non-secretor (F.D.T.).

Table 28 Synsorb Lewis-b for adhesin purification: inhibition of adhesion of C. albicans GDH 2346 to buccal epithelial cells by EPM fractions

Pretreatment of EPM	Mean no. of adherent yeasts/ 100 epithelial cells ± SEM	Percentage inhibition of adherence	AII[2]
Lewis-b bound[1]	214 ± 17	71	17.7
Lewis-b eluted[1]	476 ± 28	35	3.7
None	388 ± 11	47	1.0
PBS Control (No EPM)	754 ± 35	0	-

[1] Samples of EPM from strain GDH 2346 (100 mg ml^{-1}; 0.2 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. Both eluted and bound fractions were lyophilized and reconstituted in distilled water and desalted on a Sephadex G-25 desalting column (Pharmacia). Appropriate material was then incubated with buccal epithelial cells (37°C for 30 min) before their use in adhesion assays.

[2] Adhesion inhibition index (AII) calculated relative to untreated EPM. The buccal cell donor was blood group A and a non-secretor (F.D.T.).

Table 29 Synsorb H-1 for adhesin purification: inhibition of adhesion of C. albicans GDH 2346 to buccal epithelial cells by EPM fractions

Pretreatment of EPM	Mean no. of adherent yeasts/ 100 epithelial cells ± SEM	Percentage inhibition of adherence	AII[2]
H-1 bound[1]	276 ± 33	63	51.3
H-1 eluted[1]	483 ± 26	26	2.3
None	377 ± 33	49	1.0
PBS Control (No EPM)	737 ± 29	0	-

[1] Samples of EPM from strain GDH 2346 (100 mg ml^{-1}; 0.2 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. Both eluted and bound fractions were lyophilized and reconstituted in distilled water and desalted on a Sephadex G-25 desalting column (Pharmacia). Appropriate material was then incubated with buccal epithelial cells (37°C for 30 min) before their use in adhesion assays.

[2] Adhesion inhibition index (AII) calculated relative to untreated EPM. The buccal cell donor was blood group A and a non-secretor (F.D.T.).

Table 30 Synsorb H-2 for adhesin purification: inhibition of adhesion of C. albicans GDH 2346 to buccal epithelial cells by EPM fractions

Pretreatment of EPM	Mean no. of adherent yeasts/ 100 epithelial cells ± SEM	Percentage inhibition of adherence	AII[2]
H-2 bound[1]	435 ± 26	41	62.2
H-2 eluted[1]	547 ± 27	25	1.7
None	400 ± 16	45	1.0
PBS Control (No EPM)	733 ± 17	0	-

[1] Samples of EPM from strain GDH 2346 (100 mg ml^{-1}; 0.2 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. Both eluted and bound fractions were lyophilized and reconstituted in distilled water and desalted on a Sephadex G-25 desalting column (Pharmacia). Appropriate material was then incubated with buccal epithelial cells (37°C for 30 min) before their use in adhesion assays.

[2] Adhesion inhibition index (AII) calculated relative to untreated EPM. The buccal cell donor was blood group A and a non-secretor (F.D.T.).

Table 31 Purification of the yeast adhesin by affinity adsorption: adhesion inhibition index values for C. albicans GDH 2346

Synsorb	Adhesion Inhibition Index (based on protein content)
Lewis-a	46.7
Lewis-b	17.7
H-1	51.3
H-2	62.2

Table 32 Purification of the yeast adhesin by affinity adsorption: adhesion inhibition index values for C. albicans GDH 2023

Synsorb	Adhesion Inhibition Index (based on protein content)
Lewis-b	30.4
H-2	53.0

17.7. Lewis-a and H-1 bound protein had AII values of 46.7 and 51.3 respectively.

3.3.2 The use of partially purified material from C. albicans GDH 2023 to inhibit adhesion

To further investigate the effectiveness of Lewis-b and H-2 bound material as inhibitors of adherence, experiments were performed in which bound EPM protein from C. albicans GDH 2023 was used to inhibit the adhesion of C. albicans GDH 2023 to buccal epithelial cells, again from a donor of blood group A (a non-secretor). The results are summarized in Table 32 and given in detail in Tables 33 and 34. Lewis-b bound material had an AII of 30.4, but H-2 bound material had an AII 53.0. This latter value is the highest AII value yet recorded for an EPM fraction from C. albicans GDH 2023.

3.3.3 Effect of purified EPM protein from C. albicans on the adhesion of a heterologous yeast

Critchley and Douglas (1987b) showed that EPM from C. albicans GDH 2346 inhibited the adhesion of C. albicans GDH 2023 to buccal epithelial cells. The fact that the reverse did not hold was taken to indicate the specific nature of the adhesion (McCourtie and Douglas, 1985). As yet however, no reports have been published on the use of purified material in these inhibition tests. In addition, the method of purification involving

Table 33 Synsorb Lewis-b adhesin purification: inhibition of adhesion of C. albicans GDH 2023 to buccal epithelial cells by EPM fractions

Pretreatment of EPM	Mean no. of adherent yeasts/ 100 epithelial cells ± SEM	Percentage inhibition of adherence	AII[2]
Lewis-b bound[1]	700 ± 41	46	30.4
Lewis-b eluted[1]	1167 ± 64	23	2.6
None	881 ± 57	42	1.0
PBS Control (No EPM)	1524 ± 54	0	-

[1] Samples of EPM from strain GDH 2023 (100 mg ml^{-1}; 0.2 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. Both eluted and bound fractions were lyophilized and reconstituted in distilled water and desalted on a Sephadex G-25 desalting column (Pharmacia). Appropriate material was then incubated with buccal epithelial cells (37°C for 30 min) before their use in adhesion assays.

[2] Adhesion inhibition index (AII) calculated relative to untreated EPM. The buccal cell donor was blood group A and a non-secretor (F.D.T.).

Table 34 Synsorb H-2 for adhesin purification: inhibition of adhesion of C. albicans GDH 2023 to buccal epithelial cells by EPM fractions

Pretreatment of EPM	Mean no. of adherent yeasts/ 100 epithelial cells ± SEM	Percentage inhibition of adherence	AII[2]
H-2 bound material[1]	1118 ± 52	28	53.0
H-2 eluted material[1]	1179 ± 30	24	2.7
None	858 ± 15	45	1.0
PBS Control (No EPM)	1548 ± 49	0	-

[1] Samples of EPM from strain GDH 2023 (100 mg ml^{-1}; 0.2 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. Both eluted and bound fractions were lyophilized and reconstituted in distilled water and desalted on a Sephadex G-25 desalting column (Pharmacia). Appropriate material was then incubated with buccal epithelial cells (37°C for 30 min) before their use in adhesion assays.

[2] Adhesion inhibition index (AII) calculated relative to untreated EPM. The buccal cell donor was blood group A and a non-secretor (F.D.T.).

chromatography on Concanavalin-A Sepharose and DEAE Cellulose (Critchley and Douglas, 1987a) was not as efective as the technique described in Section 3.2, since the AII value determined for material purified from the H-2 matrix exceeded 62, in contrast to the Concanavalin-A/DEAE Cellulose protein which had an AII of just over 30.

In subsequent experiments, the results of which are shown in Table 35 and Table 36, purified material was used to inhibit heterologous yeast adhesion. Purified material from C. albicans GDH 2346 was able to inhibit the adhesion of C. albicans GDH 2023 (Table 35). The AII of 57.8 was comparable with that of purified EPM from C. albicans GDH 2023, in the inhibition of adhesion of C. albicans GDH 2023 (Table 34).

By contrast to the work of Critchley and Douglas (1987b), where only crude material was used, purified material from C. albicans GDH 2023 was able to inhibit adhesion of C. albicans GDH 2346 to buccal cells (Table 36). This result was unexpected, as although EPM from C. albicans GDH 2346 may inhibit the adhesion of C. albicans GDH 2023, the reverse is not true. EPM from C. albicans GDH 2023 cannot inhibit the adhesion of C. albicans GDH 2346. This has been taken by McCourtie and Douglas (1985) and Critchley and Douglas (1987b) as showing the specificity of yeast adhesion. Here the results show a purified fraction of the EPM from C. albicans GDH 2023

Table 35 Effect of EPM protein from C. albicans GDH 2346, purified by Synsorb H-2 batch affinity adsorption, on the adhesion of C. albicans GDH 2023

Method used to pretreat epithelial cells[1]	Mean no. of adherent yeasts/ 100 epithelial cells ± SEM	Percentage inhibition of adherence	AII[2]
H-2 bound material	652 ± 37	41	57.8
H-2 eluted material	963 ± 41	12	0.6
Untreated EPM	857 ± 20	22	1.0
PBS	1097 ± 35	0	-
Untreated EPM from GDH 2023 against C. albicans GDH 2023 adhesion	582 ± 20	47	-

[1] Samples of EPM (100 mg ml^{-1}; 0.2 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix, and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. Both eluted and bound fractions were lyophilized and reconstituted in distilled water and desalted on a Sephadex G-25 desalting column (Pharmacia). Appropriate material was then incorporated into the adherence assay.

[2] Adhesion inhibition index (AII) calculated relative to the untreated EPM.

The buccal cell donor was of blood group A, and a non-secretor (F.D.T.).

Table 36 Effect of EPM protein from C. albicans GDH 2023, purified by Synsorb H-2 batch affinity adsorption, on the adhesion of C. albicans GDH 2346

Method used to pretreat epithelial cells[1]	Mean no. of adherent yeasts/ 100 epithelial cells ± SEM	Percentage inhibition of adherence	AII[2]
H-2 bound material	519 ± 26	27	31.0
H-2 eluted material	695 ± 22	2	0.0
Untreated EPM	705 ± 19	0	-
PBS	706 ± 26	0	-
Untreated EPM from GDH 2346 against C. albicans GDH 2346 adhesion	387 ± 26	45	-

[1] Samples of EPM (100 mg ml^{-1}; 0.2 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix, and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. Both eluted and bound fractions were lyophilized and reconstituted in distilled water and desalted on a Sephadex G-25 desalting column (Pharmacia). Appropriate material was then incorporated into the adherence assay.

[2] Adhesion inhibition index (AII) calculated relative to the untreated EPM.

The buccal cell donor was of blood group A, and a non-secretor (F.D.T.).

inhibiting the adhesion of C. albicans GDH 2346. No adequate explanation for these results has yet emerged.

3.4 Development of a purification protocol for the yeast adhesin

3.4.1 Purification of the yeast adhesin by chemical and enzymic degradation prior to Synsorb H-2 batch affinity adsorption

Previous experiments (Section 3.1.2) indicated that papain digestion, followed by mild alkali treatment of EPM from C. albicans GDH 2346 produced a more effective inhibitor of yeast adhesion than crude EPM. Similarly, partially purified proteinaceous material from an H-2 matrix was shown in Section 3.3.1 (and Table 30) to be a better inhibitor of adhesion than was crude EPM.

Thus, a combination of these treatments was performed prior to affinity adsorption. From Table 37 it can be seen that the resultant protein fraction had an AII of greater than 114, and inhibited adhesion by 79%. This was the highest level of inhibition reported to date from this laboratory. In a subsequent dose response experiment, progressively increasing concentrations of proteinaceous material treated with papain, mild alkali and then bound to and eluted from the H-2 matrix, were used to pretreat the buccal cells. The results (Table 38) showed that the maximum level of inhibition achieved was approximately 65%.

Table 37 Purification of the yeast adhesin of C. albicans GDH 2346, by papain, mild alkali pretreatment prior to Synsorb H-2 batch affinity adsorption

Pretreatment of EPM	Mean no. of adherent yeasts/ 100 epithelial cells ± SEM	Percentage inhibition of adherence	AII[3]
None (H-2 bound)[1]	213 ± 16	72	55.7
None (H-2 eluted)[1]	735 ± 23	4	0.3
None	401 ± 23	48	1.0
Papain; mild alkali	278 ± 14	64	-
Papain; mild alkali[2] (H-2 bound)	158 ± 8	79	114.6
Papain; mild alkali[2] (H-2 eluted)	566 ± 31	26	2.6
PBS Control (No EPM)	765 ± 14	0	-

[1] Samples of EPM (100 mg ml^{-1}; 0.2 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix, and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. Both eluted and bound fractions were lyophilized and reconstituted in distilled water and desalted on a Sephadex G-25 desalting column (Pharmacia). Appropriate material was then incorporated into the adherence assay.

[2] EPM was pretreated with papain, followed by mild alkali prior to application to the H-2 matrix.

[3] Adhesion inhibition index (AII) calculated relative to the untreated EPM.

The buccal cell donor was of blood group A, and a non-secretor (F.D.T.).

Table 38 Purification of the yeast adhesin from EPM of C. albicans GDH 2346, by papain, mild alkali pretreatment prior to Synsorb H-2 batch affinity adsorption: effect of increasing protein concentration

Pretreatment of EPM	Mean no. of adherent yeasts/100 epithelial cells ± SEM	Percentage inhibition of adherence[1]
Papain; mild alkali		
H-2 bound ($\mu g\ ml^{-1}$)		
0.77	721 ± 27	1
1.54	641 ± 36	12
3.08	494 ± 20	32
6.15	394 ± 25	46
12.3	253 ± 14	65
Papain; mild alkali	260 ± 26	64
Crude EPM (10 $mg\ ml^{-1}$)	396 ± 15	46
PBS Control (No EPM)	765 ± 14	0

[1] Inhibition relative to the PBS control.

EPM was pretreated with papain, followed by mild alkali, prior to application to the H-2 matrix. Samples (10 $mg\ ml^{-1}$; 1.0 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix, and tumbled end-over-end at 4°C for 2 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. The bound fraction was lyophilized and reconstituted in distilled water and desalted on a Sephadex G-25 desalting column (Pharmacia), and assayed for protein (Lowry method). Appropriate material was then incorporated into the adherence assay.

The buccal cell donor was of blood group A, and a non-secretor (F.D.T.).

Experiments described by Tarantino et al. (1985) have showed that N-glycanase, purified from Streptomyces griseus, has the capacity to degrade glycoproteins into their constituent carbohydrate and protein components by cleaving the N-glycoside linkage between N-acetyl-D-glucosamine and the protein. This occurs without disrupting the integrity of the proteinaceous portion of the molecule. Since EPM from C. albicans is known to consist mainly of mannoprotein, the effect of N-glycanase on EPM from C. albicans was investigated. After N-glycanase digestion, the EPM hydrolysate was applied to the H-2 affinity matrix, eluted and used in adhesion assays.

The results of such an experiment are shown in Table 39; from this, and by comparison with Table 37, it can be seen that the AII of greater than 135 was the highest value recorded to date. Despite these treatments, elimination of all the carbohydrate from the EPM had not yet been achieved.

To this end N-glycanase followed by mild alkali treatment of the EPM was used. Although this had the desired effect of reducing the carbohydrate content of the bound fraction, some carbohydrate still remained (Table 40). The AII was fractionally greater (144.1) than that recorded previously (Table 39).

In a final attempt to remove all traces of the carbohydrate, two different pretreatments were tested.

Table 39 Purification of the yeast adhesin from EPM of C. albicans GDH 2346, by Synsorb H-2 batch affinity adsorption; effect of N-glycanase pretreatment on the adherence of C. albicans GDH 2346 to buccal epithelial cells

Pretreatment of buccal epithelial cells	Mean no. of adherent yeasts/ 100 epithelial cells ± SEM	Percentage inhibition of adherence[2]	AII[3]
H-2 bound[1]	346 ± 22	74	135.8
H-2 unbound[1]	1142 ± 53	16	3.9
Crude EPM ($10 mg ml^{-1}$)	736 ± 51	46	1.0
PBS Control (No EPM)	1356 ± 35	0	-

[1] Samples ($100 mg ml^{-1}$; 0.05 ml) were pretreated with N-glycanase for 24 h and then applied to 0.16 ml of the immobilized oligosaccharide matrix, and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. Both fractions were lyophilized and reconstituted in distilled water, desalted on a Sephadex G-25 desalting column (Pharmacia), and assayed for protein (Lowry method). Appropriate material was then incorporated into the adherence assay.

[2] Inhibition relative to PBS control.

[3] Adhesion inhibition index (AII) calculated relative to the untreated EPM.

The buccal cell donor was of blood group A and a non- secretor (F.D.T.).

Table 40 Purification of the yeast adhesin from EPM of C. albicans GDH 2346, by Synsorb H-2 batch affinity adsorption; effect of N-glycanase, mild alkali pretreatment on the adherence of C. albicans GDH 2346 to buccal epithelial cells

Pretreatment of buccal epithelial cells	Mean no. of adherent yeasts/ 100 epithelial cells ± SEM	Percentage inhibition of adherence[2]	AII[3]
H-2 bound[1]	267 ± 20	77	144.1
H-2 unbound[1]	947 ± 48	18	5.9
Crude EPM ($10 mg ml^{-1}$)	638 ± 34	45	1.0
PBS Control (No EPM)	1150 ± 51	0	-

[1] Samples ($100 mg ml^{-1}$; 0.05 ml) were pretreated with N-glycanase for 16 h and then mild alkali (0.1 M NaOH). They were than applied to 0.16 ml of the immobilized oligosaccharide matrix, and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non- specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. Both fractions were lyophilized and reconstituted in distilled water, desalted on a Sephadex G-25 desalting column (Pharmacia), and assayed for protein (Lowry method). Appropriate material was then incorporated into the adherence assay.

[2] Inhibition relative to PBS control.

[3] Adhesion inhibition index (AII) calculated relative to the untreated EPM.

The buccal cell donor was of blood group A and a non-secretor (F.D.T.).

In one method EPM was treated with either N-glycanase then papain, whilst in the second, N-glycanase/papain treatment was followed by a 24 h degradation with mild alkali. The results of these experiments are shown in Table 41. N-glycanase/papain/mild alkali treatment resulted in a fraction that was over 200 times more effective than was crude EPM.

A summary of these results, together with the corresponding biochemical analyses are shown in Table 42. This table indicates that as the protein: carbohydrate ratio decreased, the effectiveness of the H-2 bound fraction as an inhibitor of adhesion was increased. This provided further evidence for the proteinaceous nature of the adhesin.

3.4.2 The number of yeast adhesin receptors on the buccal epithelial cell surface

The number of yeast receptors on the buccal cell surface is of some interest. To investigate this, the effect of an increasing concentration of purified adhesin on the inhibition of adhesion of C. albicans GDH 2346 to buccal epithelial cells was examined. A dose response experiment was conducted with purified material adjusted to a known protein concentration (Table 43). The maximum inhibition observed was 78%. This suggests that either insufficient adhesin was used, or that more than one yeast receptor operates.

Table 41 Purification of the yeast adhesin from EPM of C. albicans GDH 2346, by Synsorb H-2 batch affinity adsorption; effect of N-glycanase, papain and mild alkali pretreatment on the adherence of C. albicans GDH 2346 to buccal epithelial cells

Pretreatment of buccal epithelial cells	Mean no. of adherent yeasts/ 100 epithelial cells ± SEM	Percentage inhibition of adherence[3]	AII[4]
H-2 bound[1]	189 ± 29	80	220.5
H-2 unbound[1]	707 ± 29	27	2.0
H-2 bound[2]	267 ± 28	72	130.8
H-2 unbound[2]	711 ± 36	28	8.5
Crude EPM ($10 mg ml^{-1}$)	508 ± 15	47	1.0
PBS Control (No EPM)	965 ± 23	0	-

[1] Samples (100 mg ml^{-1}; 0.05 ml) were pretreated with N-glycanase for 24 h, then papain (30 min) and then mild alkali (0.1 M NaOH) for 24 h.

[2] Samples (100 mg ml^{-1}; 0.05 ml) were pretreated with N-glycanase for 24 h and then papain (30 min). Samples were applied to 0.16 ml of the immobilized oligosaccharide matrix, and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. All fractions were lyophilized and reconstituted in distilled water desalted on a Sephadex G-25 desalting column (Pharmacia), and assayed for protein (Lowry method).

[3] Inhibition relative to PBS control.

[4] Adhesion inhibition index (AII) calculated relative to the untreated EPM.

The buccal cell donor was of blood group A, and a non-secretor (F.D.T.).

Table 42 Purification of the yeast adhesin from EPM of C. albicans GDH 2346, by Synsorb H-2 batch affinity adsorption; effect of decreasing protein:carbohydrate ratio

	Biochemical content ($\mu g\ ml^{-1}$)		AII[1]	Protein:
	protein	carbohydrate		carbohydrate
Crude EPM (10 mg ml^{-1})[2]	720	7920	1	1:11
H-2 bound	12.5	20.2	55.7	1: 1.62
Papain, mild alkali treated EPM: H-2 bound	6.84	7.85	114.6	1: 1.15
N-glycanase treated EPM: H-2 bound	10.54	9.24	135.8	1: 0.88
N-glycanase, mild alkali treated EPM: H-2 bound	10.70	4.18	144.1	1: 0.39
N-glycanase, papain treated EPM: H-2 bound	10.20	N	130.8	1: 0
N-glycanase, papain, mild alkali treated EPM: H-2 bound	6.72	N	220.5	1: 0

[1] Adherence inhibition index (AII) calculated relative to untreated EPM.

The buccal cell donor was of blood group A, and a non-secretor (F.D.T.).

N - Not detectable.

[2] Different crude EPM preparations were used during the purification of the yeast adhesin

Table 43 Purification of the yeast adhesin from EPM of C. albicans GDH 2346, by N-glycanase, papain and mild alkali pretreatment prior to Synsorb H-2 batch affinity adsorption: effect of increasing protein concentration

Pretreatment of buccal epithelial cells	Mean no. of adherent yeasts/100 epithelial cells ± SEM	Percentage inhibition of adherence[2]
N-glycanase, papain, mild alkali, H-2 bound (μg ml^{-1})[1]		
1.25	725 ± 19	4
2.50	625 ± 28	17
5.00	280 ± 27	63
7.50	209 ± 19	72
10.00	195 ± 17	78
Crude EPM (10 mg ml^{-1})	412 ± 15	46
PBS Control (No EPM)	752 ± 28	0

[1] Samples (100 mg ml^{-1}; 0.05 ml) were pretreated with N-glycanase for 24 h, then papain (30 min) and then mild alkali (0.1 M NaOH). All samples were applied to 0.16 ml of the immobilized oligosaccharide matrix, and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. All fractions were lyophilized and reconstituted in distilled water, and assayed for protein (Lowry method), and the protein concentration was adjusted.

[2] Inhibition relative to the PBS control.

The buccal cell donor was of blood group A, and a non-secretor (F.D.T.).

3.4.3 Use of purified adhesin to inhibit adhesion of C. albicans GDH 2346 to buccal epithelial cells

3.4.3.1 Effect of different growth conditions for C. albicans GDH 2346: throughout the experiments described in Sections 2 and 3, buccal cells from one donor and yeasts (C. albicans GDH 2346) grown in the same medium (YNB + 500 mM galactose) were used in inhibition assays. Here the growth conditions were modified to determine if there was any discernible effect. The results shown in Table 44 relate to C. albicans GDH 2346 grown in YNB + 50 mM glucose. As described in Section 1.2, yeasts with this carbon source are approximately ten times less adherent to buccal cells than are the same organisms grown with 500 mM galactose as the carbon source. For this reason, a higher concentration of 1 x 10^8 organisms ml^{-1} was used. Despite this, the purified yeast adhesin was unable to inhibit adhesion completely with inhibition levelling off at approximately 75% (Table 44).

3.4.3.2 Effect of different buccal epithelial cell donors: in all previous experiments describing inhibition by purified adhesin, a single buccal cell donor - a non-secretor of blood group A antigens in body fluids - was used. In a final purification experiment (Table 45) a different buccal cell donor - a secretor of blood group A antigens - was investigated. Although very

Table 44 Effect of increasing concentrations of protein, treated with N-glycanase, papain and mild alkali prior to Synsorb H-2 batch affinity adsorption on the adhesion of C. albicans GDH 2346 grown with 50 mM glucose as the carbon source

Concentration of purified adhesin	Mean no. of adherent yeasts/100 epithelial cells ± SEM	Percentage inhibition of adherence[1]
N-glycanase, papain, mild alkali, H-2 bound ($\mu g\ ml^{-1}$)[1]		
0.9375	384 ± 21	1
1.875	361 ± 18	7
3.75	281 ± 37	27
7.50	151 ± 19	61
15.00	97 ± 9	75
Crude EPM (10 mg ml^{-1})	326 ± 22	16
PBS Control (No EPM)	387 ± 14	0

[1] Inhibition relative to PBS control.

Assays were performed three times in triplicate, using 1×10^8 yeasts ml^{-1}.

Assays were performed using 24 h cultures of Candida albicans GDH 2346 grown in Yeast Nitrogen Base (Difco) medium containing 50 mM Glucose as the carbon source. The buccal cell donor was of blood group A, and a non-secretor (F.D.T.).

Table 45 Effect of different pretreatments on the adherence of C. albicans GDH 2346 to buccal epithelial cells from a donor who was a secretor of blood group A antigens

Pretreatment of buccal epithelial cells	Mean no. of adherent yeasts/ 100 epithelial cells ± SEM	Percentage inhibition of adherence[2]	AII[3]
PBS Control (No EPM)	768 ± 16	0	-
Crude EPM ($10mgml^{-1}$)	377 ± 22	51	1.0
H-2 unbound[1]	632 ± 45	18	11.8
H-2 bound[1]	245 ± 32	69	273.3

[1] Samples (100 mg ml^{-1}; 0.2 ml) were applied to 0.16 ml of the immobilized oligosaccharide matrix, and tumbled end-over-end at 4°C for 3 h. The matrix was eluted with PBS to remove non-specifically bound material. Bound material was eluted with a 1.5% solution of NH_4OH in sterile saline. Both fractions were lyophilized and reconstituted in distilled water, desalted on a Sephadex G-25 desalting column (Pharmacia), and assayed for protein (Lowry method). Appropriate material was then incorporated into the adherence assay.

[2] Inhibition relative to PBS control.

[3] Adhesion inhibition index calculated relative to the untreated EPM.

The buccal cell donor was of blood group A, and a non-secretor (H.S.).

little purification of adhesin had been employed (i.e. only H-2 affinity adsorption, but no chemical and enzymic dissection), the resultant AII of over 270 was the highest described throughout this study. This indicated that there was a significant difference between the effectiveness of the adhesin with respect to two donors of the same blood group but differing secretor status.

3.5 Purification protocol for the yeast adhesin

The final purification protocol developed in this study is outlined in Table 46. As was shown in Table 42, N-Glycanase, papain, mild alkali-treated EPM was totally devoid of carbohydrate and had the highest AII value (>220) recorded to date, i.e. being 220 times more effective than crude EPM as an inhibitor of yeast

3.6 Analysis of the purified yeast adhesin

Exhaustive attempts were made to demonstrate the purified adhesin as one or more bands on SDS-PAGE gels, but no success was achieved. However using F.P.L.C. it was possible to confirm the presence of the yeast adhesin and a trace from a reverse phase hydrophobicity column is shown in Figure 10.

Table 46 Purification protocol for the yeast adhesin

EPM

|

V

N-Glycanase

Papain

Mild alkali

|

V

H_2 Synsorb affinity adsorption

|

V

Purified yeast adhesin

|

V

Use in adhesion assay

Details of this protocol are supplied in Materials and Methods.

Figure 10 F.P.L.C. trace of purified yeast adhesin, on PEP HR5/5 reverse phase hydrophobicity column

100
0
mM acetonitrile (— — —)
A_{280} (——)

4 BUCCAL CELL RECEPTORS FOR CANDIDA ALBICANS

4.1 Effect of blood group and secretor status on yeast adhesion to human buccal epithelial cells

In the present study, the effect of the buccal cell donor's blood group and secretor status on the adhesion of C. albicans to buccal cells in vitro was investigated. Initially, adhesion of C. albicans GDH 2346 to buccal epithelial cells from donors of two different blood groups, A and O, was compared (Table 47). There was no significant difference between adhesion to group-A and group-O cells either for secretors or for non-secretors. However within each blood group, adhesion to cells from secretors was significantly lower than that to cells from non-secretors ($P < 0.02$ for group A; and $P < 0.05$ for group O). With group-O donors, for example 751 ± 31 yeasts (mean ± SE) adhered to 100 secretor cells as compared with 864 ± 41 yeasts which adhered to 100 non-secretor cells.

4.2 Effect of saliva and antiserum on yeast adherence

4.2.1 Effect of saliva on yeast adherence

Unstimulated whole saliva was collected, boiled for twenty minutes, clarified by centrifugation, and then added to adhesion assay mixtures. Table 48 shows the results of an experiment where the effect of saliva on yeast adherence to buccal cells from a donor of blood

Table 47 Adherence of Candida albicans GDH 2346 to buccal epithelial cells from donors of blood groups A and O: effect of secretor status

Blood group	Secretor status	Mean no. of adherent yeasts/100 BECs ± SEM	P[1]
O	Secretor	751 ± 31	
O	Non-secretor	864 ± 41	<0.05
A	Secretor	739 ± 25	
A	Non-secretor	813 ± 15	<0.02

[1] Probability values relative to the secretor cells.

Adherence assays were performed using 1×10^7 yeast cells ml^{-1}.

Table 48 Effect of saliva on the adherence of C. albicans GDH 2346 to buccal epithelial cells from a donor of blood group A (non-secretor)

Saliva treatment	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	P[2]
None (PBS Control)	813 ± 15	100	
Non-secretor saliva	759 ± 13	93	<0.01
Secretor saliva	749 ± 15	92	<0.01

[1] Adherence relative to that of controls resuspended in PBS.

[2] Probability values relative to the adhesion of PBS control.

Saliva was collected from healthy male donors (blood group A, F.D.T., H.S.), of known secretor status.

Results represent means of three independent determinations performed in triplicate.

The buccal cell donor was of blood group A, non-secretor (F.D.T.).

group A, non-secretor, was examined. Saliva from both a secretor and non-secretor (group A) had a significant inhibitory effect on yeast adhesion, with adherence being 92% and 93% ($P < 0.01$) relative to the control respectively. By contrast, Table 49 shows the results for a group A donor who was a secretor of blood-group antigens; neither saliva sample had a significant effect on yeast adhesion.

Group O individuals have been demonstrated to be more susceptible to carriage of Candida (Burford-Mason et al., 1988). Table 50 shows the effect of saliva on adherence of C. albicans GDH 2346 to buccal cells from a group O donor (secretor). Although secretor saliva had no effect, saliva from a non-secretor had a highly significant effect, enhancing adhesion by 22% ($P < 0.001$). With cells from a non-secretor, self saliva again had no effect but secretor saliva inhibited adherence by 21% (Table 51; $P < 0.001$).

These results suggest that the Lewis-b antigen in secretor saliva somehow blocks adhesion to non-secretor cells, by binding to the yeast or epithelial cell. However the Lewis-a antigen present in non-secretor saliva may enhance adhesion by acting as a bridge to link yeast and buccal cells. Non-secretor cells would already possess bound Lewis-a antigen. If this were to be blocked using the appropriate antiserum, yeast adhesion should be inhibited. This possibility was examined for

Table 49 Effect of saliva on the adherence of C. albicans GDH 2346 to buccal epithelial cells from a donor of blood group A (secretor)

Saliva treatment	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	P[2]
None (PBS Control)	739 ± 25	100	
Secretor saliva	749 ± 27	101	NS
Non-secretor saliva	778 ± 20	105	NS

[1] Adherence relative to that of controls resuspended in PBS.

[2] Probability values relative to the adhesion of PBS control; NS, not significant.

Saliva was collected from healthy male donors (blood group A, F.D.T., H.S.), of known secretor status.

Results represent means of three independent determinations performed in triplicate.

The buccal cell donor was of blood group A, secretor (H.S.).

Table 50 Effect of saliva on the adherence of C. albicans GDH 2346 to buccal epithelial cells from a donor of blood group O (secretor)

Saliva treatment	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	P[2]
None (PBS Control)	751 ± 31	100	
Secretor saliva	744 ± 30	99	NS
Non-secretor saliva	915 ± 22	122	<0.001

[1] Adherence relative to that of controls resuspended in PBS.

[2] Probability values relative to the adhesion of PBS control; NS, not significant.

Saliva was collected from healthy male donors (blood group O; F.F.C., A.E.), of known secretor status.

Results represent means of three independent determinations performed in triplicate.

The buccal cell donor was blood group O, secretor (F.F.C.).

Table 51 Effect of saliva on the adherence of C. albicans GDH 2346 to buccal epithelial cells from a donor of blood group O (non-secretor)

Saliva treatment	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	P[2]
None (PBS Control)	864 ± 41	100	
Non-secretor saliva	838 ± 26	97	NS
Secretor saliva	685 ± 29	79	<0.001

[1] Adherence relative to that of controls resuspended in PBS.

[2] Probability values relative to the adhesion of PBS control; NS, not significant.

Saliva was collected from healthy male donors (blood group O; F.F.C., A.E.).

Results represent means of three independent determinations performed in triplicate.

The buccal cell donor was of blood group O, non-secretor (A.E.).

donors of blood groups A (Table 52) and O (Table 53), both secretors and non-secretors. For each blood group a consistent pattern of results was recorded. Pretreatment of non-secretor buccal cells with anti-Lewis a antiserum had a significant ($P < 0.001$) inhibitory effect on adhesion. By contrast none of the other pretreatments produced statistically significant differences.

4.2.2 Effect of monoclonal antiserum and lectin specific for blood group antigens on yeast adherence

In further experiments anti-A monoclonal antiserum and anti-H lectin (Ulex europaeus) were used to pretreat buccal cells prior to their use in adhesion assays.

For group A donors, there was no statistically significant effect with anti-A monoclonal antiserum and the secretor cells (Table 54). By contrast, anti-A monoclonal antiserum enhanced yeast adhesion to buccal cells of the non-secretor. Similarly for group O donors (Table 55) anti-H lectin enhanced yeast adhesion by 14% to the non-secretor, but had no significant effect with the secretor. These results suggest that the monoclonal antibodies, although specific for the A antigen, are probably similar to lectins (e.g. Concanavalin A) in being able to bind to more than one site.

Table 52 Effect of anti-Lewis a and anti-Lewis b antiserum on the adherence of C. albicans GDH 2346 to buccal epithelial cells from donors of blood group A, but differing secretor status

Pretreatment of buccal cells	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[3]	P[4]
Non-secretor[1]			
None (PBS Control)	890 ± 26	100	
Anti-Lewis a	675 ± 26	76	<0.001
Anti-Lewis b	825 ± 38	92	NS
Secretor[2]			
None (PBS Control)	793 ± 41	100	
Anti-Lewis a	816 ± 19	103	NS
Anti-Lewis b	837 ± 22	106	NS

[1] The buccal cell donor was of blood group A, non-secretor (F.D.T.).

[2] The buccal cell donor was of blood group A, secretor (H.S.).

[3] Adherence relative to that of yeasts pretreated with PBS.

[4] Probability values relative to the adhesion of PBS control; NS, not significant.

Results represent means of three independent determinations performed in triplicate.

Table 53 Effect of anti-Lewis a and anti-Lewis b antiserum on the adherence of C. albicans GDH 2346 to buccal epithelial cells from donors of blood group O, but differing secretor status

Pretreatment of buccal cells	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[3]	P[4]
Non-secretor[1]			
None (PBS Control)	877 ± 23	100	
Anti-Lewis a	536 ± 12	61	<0.001
Anti-Lewis b	813 ± 22	93	NS
Secretor[2]			
None (PBS Control)	771 ± 24	100	
Anti-Lewis a	747 ± 14	97	NS
Anti-Lewis b	742 ± 17	96	NS

[1] The buccal cell donor was of blood group O, non-secretor (A.E.).

[2] The buccal cell donor was of blood group O, secretor (E.A.).

[3] Adherence relative to that of yeasts pretreated with PBS.

[4] Probability values relative to the adhesion of PBS control; NS, not significant.

Results represent means of three independent determinations performed in triplicate.

Table 54 Effect of antiserum on the adherence of C. albicans GDH 2346 to buccal epithelial cells from donors of blood group A, but differing secretor status

Antiserum pretreatment of buccal cells	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[3]	P[4]
Non-secretor[1]			
None (PBS Control)	779 ± 41	100	
Anti-A	908 ± 36	117	<0.01
Secretor[2]			
None (PBS Control)	732 ± 43	100	
Anti-A	713 ± 53	97	NS

[1] The buccal cell donor was of blood group A, non-secretor (F.D.T.).

[2] The buccal cell donor was of blood group A, secretor (H.S.).

[3] Adherence relative to that of yeasts pretreated with PBS.

[4] Probability value relative to the adhesion of PBS control; NS, not significant.

Appropriately diluted (1:64) monoclonal anti-A was used to pretreat BECs for 30 min prior to incorporation in adherence assays.

Results represent means of three independent determinations performed in triplicate.

Table 55 Effect of anti-H lectin (Ulex europaeus) on the adherence of C. albicans GDH 2346 to buccal epithelial cells, from donors of blood group O but differing secretor status

Lectin pretreatment of buccal cells	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[3]	P[4]
Non-secretor[1]			
None (PBS Control)	851 ± 29	100	
Anti-H	976 ± 49	114	<0.01
Secretor[2]			
None (PBS Control)	854 ± 39	100	
Anti-H	916 ± 45	107	NS

[1] The buccal cell donor was of blood group O, non-secretor (A.E.).

[2] The buccal cell donor was of blood group O, secretor (F.F.C.).

[3] Adherence relative to that of yeasts pretreated with PBS.

[4] Probability value relative to the adhesion of PBS control; NS, not significant.

Appropriately diluted (1:2) lectin was used to pretreat BECs for 30 min prior to incorporation in adherence assays.

Results represent means of three independent determinations performed in triplicate.

4.2.3 Effect of antiserum concentration on yeast adherence

The effect of decreasing concentrations of anti-Lewis a monoclonal on yeast adhesion was investigated with non-secretors of two blood groups, A (Table 56) and O (Table 57). For each blood group, undiluted antiserum had the most inhibitory effect and 1:2 diluted antiserum had the least effect. In addition there was a significant difference between undiluted and 1:2 diluted antiserum. Thus, although undiluted anti-Lewis a antiserum inhibited yeast adhesion to buccal cells from a group O donor by 34% and adherence to a group A donor by 33%, antiserum diluted 1:2 in PBS inhibited adhesion by only 28% and 21% respectively.

These data suggest that the Lewis-a receptor is not the major epithelial receptor, and that there is likely to be an alternative mechanism of Candida adhesion, otherwise a higher level of adhesion inhibition would have been achieved using undiluted antiserum.

4.2.4 Effect of washing buccal cells prior to pretreatment with anti-Lewis a antiserum

Blackwell (1989) proposed that the Lewis-a antigen bound to the buccal cell surface and thus enabled a bridging link to be formed when the yeast cell bound to the buccal cell/antigen complex. This could imply that successive washes would remove the antigen, and this possibility was examined. The results for such

Table 56 Effect of anti-Lewis a antiserum on the adherence of C. albicans GDH 2346 to buccal epithelial cells from a donor of blood group A (non-secretor)

Pretreatment of buccal cells	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	p[2]
None (PBS Control)	791 ± 25	100	
Anti-Lewis a (undiluted)	527 ± 20	67	<0.001
Anti-Lewis a (1:1 dilution)	601 ± 23	76	<0.001
Anti-Lewis a (1:2 dilution)	632 ± 26	79	<0.001

[1] Adherence relative to that of yeasts pretreated with PBS.

[2] Probability values relative to the adhesion of PBS control.

The buccal cell donor was of blood group A, non-secretor (F.D.T.).

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using $1x10^7$ yeasts ml^{-1}.

Table 57 Effect of anti-Lewis a antiserum on the adherence of C. albicans GDH 2346 to buccal epithelial cells from a donor of blood group O (non-secretor)

Pretreatment of buccal cells	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	P[2]
None (PBS Control)	856 ± 12	100	
Anti-Lewis a (undiluted)	563 ± 17	66	<0.001
Anti-Lewis a (1:1 dilution)	601 ± 20	70	<0.001
Anti-Lewis a (1:2 dilution)	614 ± 10	72	<0.001

[1] Adherence relative to that of yeasts pretreated with PBS.

[2] Probability values relative to the adhesion of PBS control.

The buccal cell donor was of blood group O, non-secretor (A.E.).

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using $1x10^7$ yeasts ml^{-1}.

experiments are shown for non-secretors in Tables 58 (group O) and 59 (group A). The results suggest that the first wash removed some Lewis-a binding sites from the buccal cell surface. As it is known that the Lewis antigen is to be found in the saliva of secretors (Lewis-b) and non-secretors (Lewis-a), unwashed cells would not have any superficial, non-specifically bound saliva removed. Thus in this instance with Lewis-a bound, adherence to unwashed cells would be higher than may have been expected.

Throughout this study, the data presented on adherence and blood grouping relate only to C. albicans GDH 2346. However a different strain, C. albicans GDH 2023, is known to have a significantly different mechanism of binding to epithelial cells (Critchley and Douglas, 1987b). In the following experiments anti-Lewis antiserum was used to pretreat group A and group O non-secretor buccal cells. By comparing the results for a group O non-secretor (Table 60) and a group A non-secretor (Table 61) it can be seen that the pattern of results was similar to that presented for C. albicans GDH 2346, in that although anti-Lewis b had no effect on yeast adherence, anti-Lewis a antiserum significantly inhibited adherence ($P < 0.001$).

Table 58 The effect of washing buccal cells prior to their pretreatment with anti-Lewis a antiserum: adherence of C. albicans GDH 2346 to buccal cells from a donor of blood group O (non-secretor)

Pretreatment of buccal cells	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[2]	P[3]
None (PBS Control)[1]	864 ± 20	100	
Anti-Lewis a (0 wash)	500 ± 25	57	<0.001
Anti-Lewis a (1 wash)	649 ± 22	75	<0.001
Anti-Lewis a (2 wash)	628 ± 26	73	<0.001

[1] Buccal cells received 2 washes but were pretreated with PBS only.

[2] Adherence relative to that of yeasts pretreated with PBS.

[3] Probability values relative to the adhesion of PBS control.

Buccal cell donor was a non-secretor of blood group antigens (A.E.).

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using $1x10^7$ yeasts ml^{-1}.

Table 59 The effect of washing buccal cells prior to their pretreatment with anti-Lewis a antiserum: adherence of C. albicans GDH 2346 to buccal cells from a donor of blood group A (non-secretor)

Pretreatment of buccal cells	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[2]	P[3]
None (PBS Control)[1]	745 ± 22	100	
Anti-Lewis a (0 wash)	465 ± 14	62	<0.001
Anti-Lewis a (1 wash)	567 ± 18	76	<0.001
Anti-Lewis a (2 washes)	607 ± 8	81	<0.001

[1] Buccal cells received 2 washes but were pretreated with PBS only.

2 Adherence relative to that of yeasts pretreated with PBS.

[3] Probability relative to that of yeasts pretreated with PBS.

Buccal cell donor was of blood group A, non-secretor (F.D.T.).

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using $1x10^7$ yeasts ml^{-1}.

Table 60 Effect of anti-Lewis antiserum on the adherence of C. albicans GDH 2023 to buccal epithelial cells from a donor of blood group O (non-secretor)

Pretreatment of buccal cells	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	P[2]
None (PBS Control)	1239 ± 16	100	
Anti-Lewis a	949 ± 32	77	<0.001
Anti-Lewis b	1212 ± 18	98	NS

[1] Adherence relative to that of yeasts pretreated with PBS.

[2] Probability value relative to the adhesion of PBS control; NS, not significant.

The buccal cell donor was of blood group O, non-secretor (A.E.).

Antiserum was diluted in sterile PBS (1:2 dilution) prior to use in pretreating buccal cells.

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using $1x10^7$ yeasts ml^{-1}.

Table 61 Effect of anti-Lewis antiserum on the adherence of C. albicans GDH 2023 to buccal epithelial cells from a donor of blood group A (non-secretor)

Pretreatment of buccal cells	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	P[2]
None (PBS Control)	1133 ± 20	100	
Anti-Lewis a	870 ± 35	77	<0.001
Anti-Lewis b	1154 ± 13	102	NS

[1] Adherence relative to that of yeasts pretreated with PBS.

[2] Probability value relative to the adhesion of PBS control; NS, not significant.

The buccal cell donor was of blood group A, non-secretor (F.D.T.).

Antiserum was diluted in sterile PBS (1:2 dilution) prior to use in pretreating buccal cells.

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using $1x10^7$ yeasts ml^{-1}.

4.3 Effect of sugars on yeast adhesion to buccal epithelial cells

4.3.1 Effect of sugars on the adhesion of C. albicans GDH 2346 to buccal epithelial cells

Sugar inhibition tests, as have already been described in Section 2.2.2, are widely used in work on Candida adhesion mechanisms (Douglas, 1987). The effects of different sugars on yeast adhesion to buccal cells from donors of two different blood groups, were examined. Table 62 shows that for a donor of blood group A (non-secretor), L-fucose (25 mg ml^{-1}) and N-acetyl-D-glucosamine gave similar degrees of inhibition (23% and 22% repectively) but that D-galactose was less effective (15% inhibition).

These results were comparable with those obtained both for a female donor of blood group A, non-secretor (Table 63: 26%, 20% and 13% for L-fucose, N-acetyl-D-glucosamine and D-galactose respectively), and those results for a donor of blood group A but a secretor of blood group antigens (Table 64: 26% inhibition with L-fucose and 24% inhibition with N-acetyl-D-glucosamine).

By contrast, for all three donors, a mixture of two or more of these sugars gave significantly better inhibition than single sugars. However, as previously shown in Section 2.2.2 (Table 17), this was not a cumulative effect. Although only tested once, a mixture

Table 62 Effect of different sugars on the adherence of C. albicans GDH 2346 to buccal epithelial cells from a male donor of blood group A (non-secretor)

Sugar present in mixture	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	P[2]
None (PBS Control)	524 ± 16	100	
L-fucose (50 mg ml^{-1})	311 ± 17	59	<0.001
L-fucose (25 mg ml^{-1})	402 ± 16	77	<0.001
D-galactose (25 mg ml^{-1})	444 ± 18	85	<0.001
N-acetyl-D-glucosamine (25 mg ml^{-1})	410 ± 20	78	<0.001
L-fucose:D-galactose: N-acetyl-D-glucosamine; 2:1:1 (25 mg ml^{-1})	211 ± 13	40	<0.001

[1] Adherence relative to that of yeasts suspended in PBS.

[2] Probability values relative to the adhesion of PBS control.

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using $1x10^7$ yeasts ml^{-1}.

The buccal cell donor was male, blood group A, non-secretor (F.D.T.).

Table 63 Effect of different sugars on the adherence of C. albicans GDH 2346 to buccal epithelial cells from a female donor of blood group A (non-secretor)

Sugar present in mixture	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	P[2]
None (PBS Control)	709 ± 41	100	
L-fucose	527 ± 30	74	<0.001
N-acetyl-D-glucosamine	572 ± 37	80	<0.02
L-fucose:N-acetyl-D-glucosamine; 1:1	433 ± 32	61	<0.001
D-galactose	622 ± 34	87	NS

[1] Adherence relative to that of yeasts suspended in PBS.

[2] Probability values relative to the adhesion of PBS control: NS, not significant.

All the sugars used were incorporated in the assay mixtures at a concentration of 25 mg ml^{-1}.

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using $1x10^7$ yeasts ml^{-1}.

The buccal cell donor was female, blood group A, non-secretor (N.R.).

Table 64 Effect of different sugars on the adherence of C. albicans GDH 2346 to buccal epithelial cells from a male donor of blood group A (secretor)

Sugar present in mixture	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	P[2]
None (PBS Control)	786 ± 23	100	
L-fucose	583 ± 36	74	<0.001
N-acetyl-D-glucosamine	596 ± 41	76	<0.001
L-fucose:N-acetyl-D-glucosamine; 1:1	521 ± 24	66	<0.001

[1] Adherence relative to that of yeasts suspended in PBS.

[2] Probability values relative to the adhesion of PBS control.

All the sugars used were incorporated in the assay mixtures at a concentration of 25 mg ml^{-1}.

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using $1x10^7$ yeasts ml^{-1}.

The buccal cell donor was male, blood group A, secretor (H.S.).

of all three sugars, L-fucose:D-galactose:N-acetyl-D-glucosamine (2:1:1), chosen to mimic the relative ratios of the sugars present in the Lewis-b antigen and found in the saliva of secretors, produced a percentage inhibition of 60% relative to the control. This exceeded the figure obtained with 50 mg ml^{-1} of L-fucose alone and was the highest sugar inhibition reported from this laboratory to date.

In a final series of experiments with group A donors, the immunodominant sugar, N-acetyl-D-galactosamine, was used. The results (Table 65) showed that this was the most inhibitory single sugar allowing only 58-59% adhesion. This inhibition was also greater than the L-fucose/N-acetyl-D-glucosamine mixture, but not greater than the complete mixture of all three sugars. Such results might be expected if the blood group A antigen, in which N-acetyl-D-galactosamine is the immunodominant sugar, could act as an epithelial receptor for C. albicans.

4.3.2 Effect of different sugars on the adherence of C. albicans to buccal epithelial cells from donors of blood group O

The effect of donor blood group on sugar inhibition tests with C. albicans GDH 2346 was investigated using a donor of blood group O who was a secretor. From Table 66 it can be seen that whilst N-acetyl-D-glucosamine produced no significant effect, L-fucose and D-galactose

Table 65 Effect of N-acetyl-D-galactosamine on the adherence of C. albicans GDH 2346 to buccal epithelial cells from donors of blood group A, but differing secretor status.

Sugar present in mixture	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[3]	P[4]
Non-secretor[1]			
None (PBS Control)	764 ± 30	100	
N-acetyl-D-galactosamine	452 ± 23	59	<0.001
Secretor[2]			
None (PBS Control)	686 ± 24	100	
N-acetyl-D-galactosamine	400 ± 25	58	<0.001

[1] The buccal cell donor was of blood group A, non-secretor (F.D.T.).

[2] The buccal cell donor was of blood group A, secretor (H.S.).

[3] Adherence relative to that of yeasts suspended in PBS.

[4] Probability values relative to the adhesion of PBS control.

The sugar used was incorporated in the assay mixtures at a concentration of 25 mg ml^{-1}.

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using $1x10^7$ yeasts ml^{-1}.

Table 66 Effect of different sugars on the adherence of C. albicans GDH 2346 to buccal epithelial cells from a donor of blood group O

Sugar present in mixture	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	P[2]
None (PBS Control)	786 ± 42	100	
L-fucose	596 ± 28	76	<0.001
N-acetyl-D-glucosamine	751 ± 35	96	NS
D-galactose	488 ± 47	62	<0.001

[1] Adherence relative to that of yeasts suspended in PBS.

[2] Probability values relative to the adhesion of PBS control; NS, not significant.

All the sugars used were incorporated in the assay mixtures at a concentration of 25 mg ml^{-1}.

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using $1x10^7$ yeasts ml^{-1}.

The buccal cell donor was of blood group O, secretor (F.F.C.).

showed a highly significant ability to inhibit adherence, by 24% and 38% respectively. By contrast, all three sugars investigated were able significantly to inhibit the adherence of C. albicans GDH 2023 (Table 67) by 20% (L-fucose), 28% N-acetyl-D-glucosamine and 31% (D-galactose).

5 CELL SURFACE HYDROPHOBICITY OF CANDIDA ALBICANS GDH 2346

The cell surface hydrophobicity of C. albicans GDH 2346 was investigated using the method described by Sweet et al. (1987). Table 68 shows that there was no significant change in cell surface hydrophobicity when cells were grown on different carbon sources, relative to control cells grown in yeast nitrogen but containing 50 mM glucose. This was consistent with several other studies performed in this laboratory, and considered supportive evidence that the adhesion of C. albicans is a specific effect.

Table 67 Effect of different sugars on the adherence of C. albicans GDH 2023 to buccal epithelial cells from a donor of blood group O

Sugar present in mixture	Mean no. of adherent yeasts/100 epithelial cells ± SEM	% Adherence[1]	P[2]
None (PBS Control)	895 ± 43	100	
L-fucose	719 ± 43	80	<0.001
N-acetyl-D-glucosamine	644 ± 31	72	<0.001
D-galactose	617 ± 59	69	<0.001

[1] Adherence relative to that of yeasts suspended in PBS.

[2] Probability values relative to the adhesion of PBS control.

All the sugars used were incorporated in the assay mixtures at a concentration of 25 mg ml^{-1}.

Results represent means of three independent determinations performed in triplicate.

Adherence assays were performed using 1 x 10^7 yeasts ml^{-1}.

The buccal cell donor was of blood group O, secretor (F.F.C.).

Table 68 Hydrophobicity of C. albicans GDH 2346, grown on different carbon sources

Growth in yeast nitrogen base containing	Percentage drop in A_{600} (±SEM)[1]	P[2]
Glucose, 50 mM	0.0	-
Glucose, 500 mM	1.3 ± 0.9	NS
Sucrose, 500 mM	2.6 ± 1.8	NS
Galactose, 500 mM	4.2 ± 2.9	NS

[1] Values are means of three independent determinations performed in triplicate.

[2] Probability values relative to the 50 mM glucose-grown control; NS, not significant.

D I S C U S S I O N

1 ADHERENCE OF *CANDIDA* TO BUCCAL EPITHELIAL CELLS

1.1 Measurement of adherence

A variety of methods have been described for determining the number of yeasts adhering to buccal epithelial cells. These fall into three distinct categories: light microscopy, where a visual count is employed; radiolabelling; and a Coulter count method. Of these, the visual count is the one most frequently adopted, and indeed this technique was used exclusively throughout this study. A quantitative visual determination of *Candida* adhesion *in vivo* was first reported by Liljemark and Gibbons (1973) who measured the adherence of *C. albicans* to buccal cells from rats. This method was subsequently modified by Kimura and Pearsall (1978) and again by Douglas *et al.* (1981). Briefly the procedure involves mixing standardized epithelial and yeast cell suspensions at 37°C, filtering the mixtures and counting stained, adherent yeasts on the filters by light microscopy. In the present study, yeasts were grown to stationary phase prior to their incorporation in the assay.

To prevent any counting bias, filters were coded and read 'blind'. For every sample that was filtered and fixed, 100 epithelial cells were counted. Although the method is tedious, adhesion to individual cells can be monitored if necessary, as can yeast-to-yeast adherence.

In most assays described in this study C. albicans was grown in yeast nitrogen base containing 500 mM galactose. This high concentration of carbohydrate has been shown (Douglas et al., 1981) to make individual yeasts more adhesive. Because of the high sugar concentration it would not be feasible to grow yeasts on labelled galactose and then use a radiometric assay, like that described by King et al. (1980) where [^{14}C] glucose was the carbon source.

Most adherence assays in this investigation were performed with 1 x 10^7 yeast cells ml^{-1}. This was used in preference to the yeast concentration of 1 x 10^8 cells ml^{-1} employed by Douglas et al. (1981) since adherence values with the latter concentration were very high and counting was difficult. A concentration of 1 x 10^7 yeast cells ml^{-1} was considered here to be more reliable and also more convenient.

In the initial studies (Results, 1) buccal epithelial cells (1 x 10^5 ml^{-1}) were obtained from a single donor who was a healthy adult male (blood group A, non-secretor of blood group antigens); the donor's cells showed no obvious or visible daily changes. Cells were collected at the same time of day, generally between 8.30 am and 10.30 am. Buccal cells were sampled where possible on Thursday, Friday and then on the following Monday, allowing the buccal mucosa time for some regeneration over the weekend. The buccal cells investigated here

were exfoliated, and collected by gentle swabbing of the inside of the cheek. Variation in adherence occurs with different donors and this will be discussed in detail later. This variation is widely documented (King et al., 1980; Kearns et al., 1983). When light microscopy is used to measure adhesion, the distribution of yeasts over the buccal cell surface can be seen. Certain epithelial cells have higher numbers of yeasts attached to them than others. This presumably arises because some cells have more yeast receptors than others.

Initially, adherence of C. albicans to buccal cells was measured following periods of incubation ranging from 15 to 90 min. This was to determine the most suitable duration for the assay. The results indicated that there was no detectable increase in adhesion after 45 min. of incubation, and so this incubation period was used throughout the study.

1.2 Adherence of different Candida species

The adherence of Candida isolates obtained from outpatients at a clinic at the Royal Dental College, Copenhagen was investigated using the standard assay. Initially all the isolates were thought to be C. albicans, and as part of the routine experimental procedure, were tested for germ-tube formation in foetal-calf serum, and for growth on glucose, sucrose and galactose. As a result of these tests four of the ten

isolates were found to be species of Candida other than C. albicans. Two were identified as C. lusitaniae whilst the other two were found to be C. glabrata and C. parapsilosis. Four strains were described as being from one patient. This is contrary to the common finding (Odds, 1988) that only one strain can be isolated from a single host, in this case the mouth of a leukoplakia sufferer. Since any organisms on the mucosal surface may circulate in the oral milieu it seems unlikely that some strains originate solely from the normal mucosa, and others from nodular mucosa. It would seem more likely that there is a higher concentration at the infected site but that organisms circulate freely in the oral cavity, unless very rigidly bound.

The results of the assays were generally similar to those of Douglas et al. (1981), McCourtie and Douglas (1984), and Critchley and Douglas (1985) which indicated that strains from active infection show enhanced adherence after growth in a medium with a high galactose content. However, strain 906 showed enhanced adhesion and was from normal mucosa. Strains 905 and 911 may have originated from leukoplakia infections but were isolated from normal mucosa.

Kearns et al. (1983) did not observe any differences in adherence between yeasts isolated from active infections and yeasts isolated from asymptomatic carriers. This is in direct contrast to the results of

Douglas et al. (1981) and Critchley and Douglas (1985), where various isolates adhered similarly after growth in glucose but differences were revealed following growth on 500 mM galactose. In the latter studies, strains from carriers showed much smaller increases in adherence after growth in a high galactose medium. These differences between the two laboratories may have resulted from the use of malt agar or Lee's medium by Kearns et al. (1983). Whilst infective strains grown in Lee's medium did adhere to buccal cells, such strains grown in the high sugar medium synthesized more adhesins and thus bound in higher numbers.

Since precise details of the site of isolation of the leukoplakia strains are not available, and it is not known whether some of the patients ever developed Candida-associated infections, only limited conclusions may be drawn from the present study. With C. albicans GDH 2346, yeasts grown in a medium containing 500 mM galactose were over 9 times more adherent to buccal cells than 50 mM glucose-grown organisms. Under the same conditions C. albicans RDC 902, 904, 905, 910 and 911 all showed significant increases in relative adhesion. All of these isolates were from patients that had oral infections. The results therefore support those of McCourtie and Douglas (1984) who concluded that there is a relationship between the ability of different Candida albicans strains to adhere to epithelial cells, their

capacity for cell-surface modification, and their virulence.

The results also indicate that C. lusitaniae has only a limited capacity to modify its cell surface and is thus less likely to be a major causal agent of Candida infections.

2 EXTRACELLULAR POLYMERIC MATERIAL (EPM) AND ITS EFFECT ON YEAST ADHESION

McCourtie and Douglas (1981) have shown that growth of C. albicans in high concentrations of galactose results in the production of an additional layer of fibrils on the cell surface. These adhesion enhancing fibrils are sloughed off into the surrounding medium during prolonged incubation of the yeast at 37°C. In the present study, cultures of C. albicans were grown for 5 d at 37°C to generate yields of EPM of approximately 15%; this figure is roughly in line with yields obtained by McCourtie and Douglas (1985) and Critchley and Douglas (1987a). Fibrils have also been described by Gardiner et al. (1982), while Pugh and Cawson (1978) noted a mucus layer on the Candida cell surface. Biochemical analysis of EPM isolated from culture supernatates indicated that the carbohydrate:protein ratio ranged from 10.7:1 in C. albicans MRL 3153 to 6.9:1 with C. albicans GDH 2023. These results confirm previous data from this laboratory. McCourtie and Douglas (1985) found that EPM contained a

large quantity of mannose and they suggested that it consisted mainly of mannoprotein.

A variety of techniques have been employed in the isolation of EPM. McCourtie and Douglas (1985) described EPM that was isolated by precipitation of culture supernatants with acetone. The method described by Critchley and Douglas (1987a) involved the extensive dialysis of culture supernates to remove low molecular-weight components; the retentate was freeze-dried. The latter method was preferred as there was no possibility of denaturation or aggregation that could have occurred during acetone precipitation. In the present study the procedure was modified to include an ultrafiltration step. This ultrafiltration speeded up the EPM production process by several days. EPM prepared in this way was found to be as effective in inhibiting adherence of C. albicans GDH 2346 to buccal cells as were the methods described above.

Previous attempts in this laboratory to resolve EPM by SDS-polyacrylamide gel electrophoresis have always proved unsuccessful (Critchley, 1986). No bands were detected when gels were stained with Coomassie blue R250 or silver stain. This was considered to be caused by the low (5 mg ml^{-1}; 25 μl) levels of glycoprotein applied to the gel, probably beyond detectable limits. However silver staining is normally useful down to nanogram level. In the present study SDS gels were run using EPM

from several C. albicans strains and several distinctive bands were visualized using both silver stain and Coomassie blue R250. Samples of EPM, prepared several years prior to the gel experiments described here, were applied to the gel and found to run identically with freshly prepared EPM; indeed this was a suitable means of verifying the similar nature of EPM samples prepared in this laboratory. One possible explanation for this inconsistency may be that previous workers did not use a high enough concentration of acrylamide in the separating gel; alternatively, the fixing protocol may have been in some way flawed and the protein bands lost during the washing phase of the experiment.

The results of affinity chromatography indicated that EPM protein possesses the ability to interact with immobilized sugars. These preliminary experiments were done to investigate whether EPM from C. albicans had any 'lectin-like' binding properties. Fractions with sugar-binding specificities were isolated by applying crude EPM preparations to Sepharose affinity columns containing a coupled sugar. The sugars coupled to the Sepharose gels were chosen because they are known to be present on the surface of host cells. Possibly the protein component of EPM binds in a 'lectin-like' way to glycoside receptors on the host cell surface. EPM from strains GDH 2346 and GRI 681 had similar sugar specificities and bound to L-fucose, D-mannose and N-acetyl-D-glucosamine at

decreasing levels of magnitude. EPM from C. albicans GDH 2023 showed a different order of sugar specificities and bound in preference to D-mannose and in approximately equal quantities to N-acetyl-D-glucosamine and L-fucose. The results described here are in broad agreement with those presented by Critchley and Douglas (1987a). Indeed these results further confirmed that the EPM samples used in these and previous experiments were similar.

Further experiments were conducted using bound protein fractions as inhibitors of adhesion against the homologous yeast strain. The use of adherence inhibition indices represents an attempt to relate adhesion inhibition to the protein content of each fraction and hence determine its relative efficiency at inhibiting adhesion. Results were compared with those of Critchley (1986) and, for both C. albicans GDH 2346 and GDH 2023, there were marked differences between the two studies. The entire protocol was repeated to ensure reproducibility; as the results were consistent (Tables 14 and 15) it was concluded that the differences must be due to the buccal cell donor. All other criteria had been standardized, namely the EPM, the sugar binding capacity of the EPM, and the adherence assay.

Evidence suggesting that epithelial cell-surface sugars function as attachment sites for some bacteria has come from experiments in which adherence was inhibited with monosaccharides, lectins, sugar oxidants and

glycosides. In the present study, sugars which may function as epithelial cell receptors were utilized to inhibit adherence to see if there was consistency with the results provided by the lectin-inhibition assays. The results provided in Tables 16 and 17 indicate that for C. albicans GDH 2346, L-fucose- and N-acetyl-D-glucosamine-containing receptors on the epithelial cell surface play a major role in adherence, whereas for C. albicans GDH 2023, D-mannose-containing receptors appear to be predominant.

This first observation, with respect to C. albicans GDH 2346 has some similarities to previously published data. L-fucose has not been detected on the surface of C. albicans and this eliminates the possibility of it acting as an adhesion analogue. Jones and Freter (1976) reported that L-fucose and other fucosides, may act as receptors for Vibrio cholerae in isolated brush-border membranes. Cinco et al. (1984) used L-fucose to inhibit adherence of Campylobacter species to epithelial cells. It is interesting to note that in these latter experiments, inhibition was enhanced by increasing sugar concentration in the assay up to 50 mg ml^{-1}. After this no further inhibition was seen, and this would suggest that another receptor may have been operating in conjunction with L-fucose.

With regard to the present study, and previously discussed results (Critchley, 1986) there were

inconsistencies. The possibility of experimental error was considered small; the most obvious difference was between buccal cell preparations. Blood-group and secretor status of the donors were considered the most likely areas of difference as previous data (Critchley, 1986) had been generated with a donor of blood group B, who was a secretor of blood group antigens. Effects caused by using buccal cells from donors of different blood group and secretor status will be discussed later. At this stage in the investigation the results indicated firstly that different individuals with different buccal cells generate different lectin-inhibition assay results, and secondly that different buccal cells give different results in sugar inhibition tests.

The effect of C. albicans lectin-like proteins on the adhesion of heterologous strains was investigated. The specificity of yeast adhesion has been demonstrated (Critchley and Douglas, 1987b) in that although EPM from C. albicans GDH 2346 inhibits adhesion of C. albicans GDH 2023, the reverse is not true. Here partially purified fractions from C. albicans GDH 2023, like the crude EPM, had no significant effect on the adhesion of C. albicans GDH 2346. By contrast, the L-fucose- and N-acetyl-D-glucosamine-specific lectins isolated from C. albicans GDH 2346 inhibited adhesion of C. albicans GDH 2023, whereas the D-mannose-specific lectin had no effect. Although these results appear to support the conclusion

that C. albicans GDH 2346 does have some predilection for L-fucose and N-acetyl-D-glucosamine residues found on the epithelial cell surface, a more detailed investigation of buccal cell donors was obviously required.

3 PURIFICATION OF THE YEAST ADHESIN

3.1 Chemical nature of the yeast adhesin

C. albicans has been reported to adhere in comparable numbers to different epithelial cell types (Botta, 1981); however it is not necessarily true that the binding mechanism is the same between C. albicans and all epithelial cells. In the present study, an attempt was made to define the binding mechanism(s) of C. albicans to buccal epithelial cells to which much of the literature relates.

Investigations carried out by other workers have focused on the use of blocking agents, enzymes or antibiotics, as this approach has been widely used in studying bacterial adhesion. To date, most experimental evidence supports a role for yeast mannoprotein in the attachment of yeasts to buccal cells. Surface mannan is important in mediating adherence of C. albicans to fibrin-platelet clots formed in vitro (Maisch and Calderone, 1981). Ray et al. (1984) concluded that cell wall mannan participated in the adhesion complex, yet found only minor inhibition of adherence using mannan.

This inconsistency may have been due to the fact that the mannan used was derived from Saccharomyces cerevisiae and not from C. albicans. As is demonstrated here, EPM mannoprotein from C. albicans inhibits yeast adhesion to buccal cells.

The adhesin mediating attachment of C. albicans is a component of the EPM produced by the yeast after growth in a medium containing high concentrations of certain sugars, most notably galactose (McCourtie and Douglas, 1985a). Several investigators have shown that yeast adhesins may be released into the culture medium (McCourtie and Douglas, 1985; Tronchin et al., 1984; Diamond et al., 1980). Sentendreu and Northcote (1968) demonstrated that exposure to dilute alkali causes β-elimination of manno-oligosaccharides attached to serine and threonine residues in yeast mannoproteins. In this study EPM preparations were subjected to varying degrees of alkaline hydrolysis. The results indicated that the most suitable concentration of mild alkali was 0.1 M NaOH. This finding ensured that in subsequent experiments deglycosylation of the EPM via β-elimination was performed with maximum efficacy, without hydrolysing the protein portion of the mannoprotein.

Evidence for the importance of protein in the yeast adhesin was derived from experiments examining the effect of EPM samples treated with papain, on the adherence of C. albicans to buccal cells. Critchley and Douglas

(1987a) showed that trypsin, chymotrypsin and pronase all reduced the ability of EPM to inhibit adherence. This suggested that these enzyme treatments degrade the adhesin or interfere with the stereochemistry of the interaction with buccal cells thus destroying the blocking effect of EPM on adhesion. Incubation of EPM with papain, on the other hand, enhanced its ability to inhibit adhesion of C. albicans to buccal cells, while a combination of papain followed by mild alkali resulted in notably increased inhibition. Here, the effect of papain/mild alkali was reproducible for two donors of the same blood group, but different secretor status. The importance of secretor status, with respect to yeast adhesion will be discussed fully in the next section. However the papain/mild alkali effect was not confined to C. albicans GDH 2346 but was also obtained with strain GDH 2023; this would imply that for two very different C. albicans strains, the method of attachment is via a proteinaceous adhesin. It was suggested that papain/mild alkali treatment releases peptide fragments capable of binding to epithelial cell receptors more efficiently than untreated EPM (Critchley and Douglas, 1987a). Such fragments may be related to the small mannoprotein reported by Lee and King (1983) to be released by treating C. albicans with papain. This mannoprotein inhibited yeast adhesin to vaginal epithelial cells. Small glycopeptides are not unknown. Diedrich et al.

(1984) showed that low molecular weight glycoprotein could be isolated from the culture medium of C. albicans; indeed, Diamond et al. (1980) isolated peptides from C. albicans hyphae which had been subjected to ultra violet light and found that they impaired neutrophil function.

As will become evident, this part of the study related to development of a protocol for the purification of the yeast adhesin. Thus it was essential to ensure the specificity of the enzymic and chemical treatments to which EPM was subjected prior to its use in the adhesion assay; to this end a series of control experiments was performed. In every case, increased inhibition by papain/mild alkali-treated EPM was attributable to the degradation of EPM and not to other components of the incubation mixture, for example the α_2 macroglobulin which inhibited papain acivity.

3.2 Purification of the yeast adhesin by affinity adsorption

Experiments carried out in this study (Results 2) indicated that it was possible to purify lectin-like adhesins from the crude EPM of C. albicans using affinity chromatography. Single sugars were immobilized on epoxy-activated Sepharose beads; these beads were packed into a column and crude EPM solution was passed over the beads. Purified bound material was then used in adherence assays. With the Synsorb adsorbents (which are

expensive), only small quantities of material were available and a column technique was impracticable. To test the efficacy of a batch, or tumble method of affinity adsorption, EPM was incubated with epoxy-activated Sepharose linked to immobilized sugars. Bound EPM was eluted using the appropriate sugar and the results indicated that the tumble technique produced data of a similar pattern to those from column chromatography.

Synsorb affinity adsorbents have blood group oligosaccharides bound to an inert silica matrix and have been used to purify highly specific lectins and antisera. These Synsorb matrices were therefore used to purify the protein from crude EPM solutions derived from three strains of C. albicans, namely GDH 2346, GDH 2023 and GRI 681. The relative binding specificities of the crude EPM solutions showed no obvious pattern between strains, although the EPM from C. albicans GDH 2023 and GRI 681 did show some predilection for the H-2 and Lewis-b matrices, respectively. The four oligosaccharides employed were all thought to be of relevance in the adhesion of C. albicans to buccal cells. Lewis-a and Lewis-b antigens are found in the saliva of non-secretors and secretors, respectively, of blood group antigens. Thus these antigens are to be found in the in vivo adhesion milieu. H-1 and H-2 antigens are precursors of the A and B antigens of the ABO blood group system. They

have structures similar to those of the Lewis-a and Lewis-b antigens (Figure 4). All these antigens are fucose-containing oligosaccharides; L-fucose has been implicated as a receptor for C. albicans GDH 2346 (Critchley and Douglas, 1987b).

3.2.1 Efficiency of partially purified adhesin from C. albicans GDH 2346 and GDH 2023 to inhibit adhesion of the homologous yeast strain

Adhesion inhibition tests were used to monitor purification of EPM components which could be used to block yeast adhesion to buccal epithelial cells. Purified material from the Synsorb H-2 matrix inhibited yeast adhesion to buccal cells 62 times more efficiently (on a protein weight basis) than did crude EPM. It was unclear whether more than one type of adhesin was present in this fraction. Experiments described by Critchley and Douglas (1987b) indicate that C. albicans GDH 2346 does possess more than one adhesin for buccal cells.

Adhesion inhibition tests were also used to monitor the purification of the yeast adhesin from C. albicans GDH 2023. As the main emphasis of this study was to purify the adhesin from strain GDH 2346, only two purified fractions were tested. Affinity binding to the H-2 matrix appeared to purify the adhesin from C. albicans GDH 2023 more than 50-fold.

3.2.2 Efficiency of partially purified adhesin from C. albicans GDH 2346 and GDH 2023 to inhibit adhesion of the heterologous yeast strain

Strain-related differences in adherence of C. albicans have been reported throughout this study; with this in mind, the effect of purified EPM protein from C. albicans on the adhesion of a heterologous yeast was investigated. C. albicans GDH 2023 has been reported to show a different pattern of response to EPM preparations from various other C. albicans strains. Adhesion of C. albicans GDH 2023 is substantially inhibited only by the homologous EPM and to a lesser extent, by that of strain GDH 2346 (Critchley and Douglas, 1987b). Moreover, crude EPM from this yeast failed to give significant inhibition of adhesion with any other C. albicans strain tested. This is consistent with the results shown in Tables 18 and 19 and would point to a different adhesion mechanism for this strain. Consequently the results of experiments described here (Table 36) in which purified EPM protein from C. albicans GDH 2023 was able to partially inhibit the adhesion of C. albicans GDH 2346 were unexpected and difficult to explain. However these data would also suggest that there is more than one binding mechanism for the yeast to the buccal cell surface.

3.3 Purification protocol for the yeast adhesin

The development of a protocol for purification of the yeast adhesin involved using information from experiments described in Results 3.1.2 and 3.3.1. In these assays, optimal conditions for mild alkaline hydrolysis, papain digestion and Synsorb H-2 adsorption were established. When a combination of papain/mild alkali/H-2 adsorption was tested, an AII value of 114 was calculated (Table 37). This was taken as evidence of an improved purification protocol. Since adhesion inhibition was 79%, it was worth considering if higher concentrations of the purified material would increase inhibition to 100%. The data presented in Table 38 show that this is not the case; indeed the maximum inhibition achieved in this assay was only 65%. Such day-to-day differences in the response of buccal cells are not uncommon. The results imply that there is not just one Candida binding mechanism per strain but at least two. However, the bound fraction did still contain some carbohydrate, and in an effort to further purify the adhesin, more degradation experiments were performed. In this case an additional deglycosylation step was introduced.

The experiments of Tarantino et al. (1985) have been referred to in Results 3.4.1. N-Glycanase, purified from Streptomyces griseus, has the capacity to degrade glycoproteins into their constituent carbohydrate and protein components, without disrupting proteinaceous

integrity. N-Glycanase digestion was performed firstly before H-2 adsorption, secondly before mild alkali treatment then H-2 adsorption, and thirdly before papain/mild alkali treatment then H-2 adsorption. Gradually the data presented in Table 42 were accumulated. During the course of the protocol shown in Table 42, the protein:carbohydrate ratio decreased, and the effectiveness of the H-2 bound fraction as an inhibitor of adhesion increased. After N-Glycanase, papain, mild alkali treatment and Synsorb H-2 adsorption there was no detectable carbohydrate and the AII was 220. As was attempted at previous stages, a dose response experiment was performed. Again maximum inhibition was not 100% but 78%; thus more than one yeast receptor probably operates.

Two additional experiments yielded interesting data. Firstly, C. albicans GDH 2346 was grown in YNB + 50mM glucose as an alternative to high galactose medium. Again the purified yeast adhesin was unable to inhibit adhesion completely, with the dose-response curve levelling off at approximately 75% inhibition. Secondly, an alternative buccal cell donor was used. The new donor was of identical blood group (A), but of different secretor status. Although only a very low concentration of partially purified adhesin was used, and the crude EPM was subjected merely to H-2 affinity adsorption rather than the complete purification protocol, an AII of over

270 was obtained (Table 45). This would suggest significant differences in the ability of such material to inhibit adhesion to buccal cells from donors of differing secretor status, as the corresponding AII value for a non-secretor was 55.7 (Table 42).

The final purification protocol is outlined in Table 46. Exhaustive attempts were made using SDS-PAGE techniques to visualize the adhesin without success. Various reasons for this failure could be suggested. It seems unlikely that the protein peptide was small enough to run off the end of the gel i.e. ahead of the tracking dye. However, it is possible that insufficient protein was applied to the gel, or that the gel was inadequately fixed. To verify the existence of the purified adhesin F.P.L.C. was utilized. A suitable trace was obtained using a A_{280} detector.

4 THE BUCCAL CELL RECEPTOR

Adhesion of Candida to epithelial cells is the first stage in the colonization of mucosal surfaces by this organism. Although the mechanism of adhesion is not fully established, there is now considerable evidence for the involvement of glycosides as epithelial receptors for the yeast (Critchley and Douglas, 1987b; Douglas, 1989). A number of studies have demonstrated that in vitro, C. albicans adheres more readily to exfoliated buccal or vaginal epithelial cells from some donors than from

others (King et al., 1980; Sobel et al., 1981), suggesting that some individuals may be more susceptible to colonization by the yeast. Recently, Burford-Mason et al. (1988) reported that oral carriage of C. albicans in healthy subjects could be correlated with two host factors, namely blood group O and non-secretion of blood group antigens, with the trend towards carriage being greatest in group O non-secretors. In the present study the effect of donor blood group and secretor status on adhesion of C. albicans to buccal epithelial cells in vitro, was investigated.

4.1 Effect of blood group and secretor status on yeast adhesion to human buccal epithelial cells

Correlations between ABO blood group or secretor status and vulnerability to disease have been documented for a number of bacterial infections (Mourant et al., 1978). With C. albicans, blood group O and non-secretion of blood-group antigens have been identified as possible risk factors for oral carriage of the yeast in healthy subjects (Burford-Mason et al., 1988). The results of the present study shown in Table 47, indicate that Candida adhesion to the oral mucosa - the first step in the colonization process - is affected by the secretor status of the host. However, in this small sample of four buccal cell donors, there was no evidence that yeast adhesion was dependent on the ABO blood group.

4.2 Effect of saliva and antiserum on yeast adherence

The possible importance of secretor status was first suggested by Blackwell et al. (1986) who briefly reported that Candida adhesion to buccal cells could be inhibited by secretor saliva but enhanced by non-secretor saliva. The results shown in this study both confirm and contradict these findings and also provide additional evidence that the Lewis-a antigen which is adsorbed on the surface of the buccal cells in non-secretors could function as a receptor for the yeast. This conclusion would be consistent with previous work from this laboratory (Critchley and Douglas, 1987b) which indicated that C. albicans GDH 2346 produces surface mannoproteins with a lectin-like affinity for L-fucose residues, and that fucose-containing glycosides are likely to be major epithelial cell receptors for this yeast strain. It would also be consistent with the protocol described here for the purification of the yeast adhesin; this involves binding an EPM digest to a Synsorb-H-2 affinity matrix containing a terminal α-fucosyl residue and assaying the adhesion inhibition index relative to the crude EPM. The Lewis-a antigen also contains a terminal L-fucose residue. Fucose is the immunodominant sugar of the H-antigen of blood group O and it is possible that cell-bound H antigen could similarly function as a receptor although its presence in secretor saliva may effectively block adhesion. The results presented here

do not suggest an analagous role for the Lewis-b antigen since treatment of buccal cells with anti-Lewis b antiserum had no effect on adhesion (Tables 52 and 53).

The effects of monoclonal antiserum specific for blood group A and anti-H lectin (Ulex europaeus), on yeast adherence suggest that these treatments bind specifically to the appropriate antigens. However, such experiments can lead to confusing results; Concanavalin A and lentil lectin both significantly enhanced the adherence of strain GDH 2346 to buccal epithelial cells (Critchley and Douglas, 1987b). This may have been due to the pH value (7.2) used in the assay, as Concanavalin A has been shown by Bittiger and Schnebli (1976) to exist as a pseudo-tetrahedral molecule at this pH, with four saccharide binding sites, one on each subunit. Yeast cell envelopes are abundant in mannoprotein which is composed of α-D-mannopyransyl residues. The mannan backbone consists of $\alpha(1-6)$ linkages with side chains varying in length from 1 to 3 monomers joined by $\alpha(1-2)$ and $\alpha(1-3)$ linkages which are recognized by Concanavalin A (Goldstein et al., 1965). In the present study no aggregation of buccal epithelial cells occurred when they were pretreated with anti-A monoclonal or anti-H lectin (Ulex europaeus).

The effect of the antiserum concentration on yeast adherence was investigated. Buccal cell donors of differing blood group were selected for these

experiments, although they had the same secretor status. By varying the concentration of the anti-Lewis a antiserum it was concluded that the Lewis-a antigen is not the major epithelial receptor, and that another mechanism must operate. Further experiments are described here that show the effect of washing on buccal cells. Blackwell (1989) proposed that the Lewis-a antigen bound to the buccal cell surface and thus a bridging link was formed when the yeast bound to the buccal cell/antigen complex. Successive washes should remove the antigen. The results of the present study indicate that the first wash removes some Lewis-a binding sites from the buccal cell surface, but not all, and this trend may or may not continue with progressive washes.

In contrast to the experiments discussed above, some assays were performed with C. albicans GDH 2023, a strain known to be significantly different in binding mechanism (Critchley and Douglas, 1987b). These assays involved pretreating group A and group O, non-secretor buccal cells with anti-Lewis a and anti-Lewis b antisera. For non-secretors, anti-Lewis a antiserum inhibited adhesion of C. albicans GDH 2023 for both group A and group O buccal cell donors, i.e. secretor sta tus influenced yeast adhesion, but changing the C. albicans strain had no effect, as the results were similar to those seen with C. albicans GDH 2346.

4.3 Evidence from sugar inhibition tests for the role of glycosides as epithelial cell receptors

In vitro sugar inhibition tests have been widely used with both bacteria and yeasts to predict the nature of epithelial cell receptors. The sugars used to block adhesion here are thought to be present on host cell surfaces in glycoproteins or glycolipids (Sharon *et al.*, 1981).

C. albicans GDH 2346 was inhibited by L-fucose; this sugar has not been detected on the cell surface of *C. albicans* which rules out the possibility of it acting as an adhesin analogue (Table 62). Jones and Freter (1976) recognized that L-fucose and other fucosides may function as receptors for *V. cholerae*. Cinco *et al.* (1984) described the adherence of *Campylobacter* species as being mediated by L-fucose. In the latter study, inhibition of *Campylobacter* adherence by L-fucose was concentration-dependent up to 50 mg ml^{-1}, with no further inhibition above this point. This suggests that another receptor may be present in addition to L-fucose. Here, results from the dose response experiments using purified yeast adhesin (Tables 42 and 43) would suggest a similar situation with *Candida* adherence.

The results of these sugar inhibition tests were comparable with those obtained both for a female donor of blood group A, non-secretor and for another male donor of blood group A but a secretor of blood group antigens.

Thus, although the blood group of the buccal cell donor influences the results of sugar inhibition tests (Tables 62 and 66), neither the sex nor the secretor status of the donor affects the results of these assays (Tables 62 to 64).

Sobel et al. (1981) noted that L-fucose inhibited the adherence of a clinical isolate of C. albicans to vaginal cells. Sanden et al. (1982) on the other hand found that methyl-α-D-mannoside inhibited adherence, whereas Segal et al. (1982) reported that amino sugars inhibited yeast adhesion to epithelial cells. In E coli, nine adhesins have been described which are proteins and interact in a lectin-like fashion with glycoside receptors on the host cell surface (Jones and Isaacson, 1983). Different C. albicans strains may also have different protein adhesins which are specific for different sugars.

Segal et al. (1982) claimed that inhibition with N-acetyl-D-glucosamine was due to its role as an adhesin analogue rather than a receptor analogue. They described chitin, a soluble derivative and N-acetyl-D-glucosamine as inhibitors of adherence when incorporated in assay mixtures. Chitin, which it was suggested mediated adhesion, is located in the bud scars of yeasts. Poulain et al. (1978), in their scheme for cell wall architecture, showed chitin not to be on the cell surface but located several layers beneath in an alkali-insoluble matrix with glucan. The inhibitory effect of

N-acetyl-D-glucosamine on the adherence of C. albicans observed by Segal et al. (1982) may therefore be due to the sugar acting as a receptor analogue, as chitin is not easily accessible for interacting with the epithelial cell receptor.

For all three donors described in Tables 62 (see also Table 17), 63 and 64 a mixture of the sugars L-fucose and N-acetyl-D-glucosamine gave significantly better inhibition than single sugars. This was not a cumulative effect; however it would suggest that the receptor for yeast adhesion is a complex of two or more sugars. Indeed when a mixture of L-fucose, D-galactose and N-acetyl-D-glucosamine was tested as an inhibitor, the percentage inhibition was the highest reported for any sugar solution from this laboratory to date. This combination of sugars was chosen at a stage in the investigation when evidence was accumulating to suggest that secretor status influenced the adhesion of yeast to buccal epithelial cells. These three sugars combine to form the Lewis blood group oligosaccharide and as virtually all humans are either Lewis a- or Lewis b-positive it was not surprising that this combination was so effective. In the final assays using group A donors N-acetyl-D-galactosamine gave greater inhibition of yeast adhesion than either L-fucose, N-acetyl-D-glucosamine or a mixture of these two sugars. Such a result might be expected if the blood group A antigen, in which N-acetyl-

D-galactosamine is the immunodominant sugar, could act as an epithelial cell receptor for C. albicans.

The effect of different sugars on the adherence of C. albicans to buccal epithelial cells from a donor of blood group O was investigated. In contrast to experiments with A donors, N-acetyl-D-glucosamine produced no significant inhibition, but L-fucose and D-galactose significantly inhibited adherence. This suggested that for individuals with different blood groups, different receptors function. This would again be consistent with previously published data if the blood group determinant on buccal cell surfaces was the receptor for C. albicans, as L-fucose is the immunodominant sugar for the H-antigen. Finally the effect of different sugars on the adherence of C. albicans GDH 2023 to buccal cells from a donor of blood group O was investigated and in contrast to strain GDH 2346, N-acetyl-D-glucosamine was found to significantly inhibit adhesion. This was consistent with the data of Critchley and Douglas (1987b). However these latter experiments were performed using a cell donor of blood group B, where D-galactose is the immunodominant sugar, and no significant inhibitory effect was noticed when D-galactose was added to adhesin assay mixtures. The reasons for these inconsistencies have yet to be determined.

In conclusion, in vitro sugar inhibition tests have been widely used to predict the nature of epithelial cell

receptors for both bacteria and yeasts. The results discussed here indicate that such tests can be influenced by the ABO blood group of the buccal cell donor. This finding should be borne in mind in future work on Candida adhesion mechanisms.

A P P E N D I C E S

I BIOCHEMICAL ASSAYS

A Carbohydrate determination using the phenol-sulphuric acid method (Dubois et al., 1956)

Reagents

1 Concentrated sulphuric acid (Reagent grade)

2 5% phenol (v/v)

Procedure

Phenol reagent (0.05 ml) was added to sample (2ml) containing 0 - 100 $\mu g\ ml^{-1}$ carbohydrate. Reagent grade concentrated sulphuric acid (5 ml) was rapidly added and samples were left for 30 min at room temperature to allow colour development. Samples were read at 485 nm against a blank of distilled water in a spectrophotometer (Shimatzu PR-1). A standard curve was produced using mannose.

B Protein determination using the Lowry method (Lowry et al., 1951)

Reagents

A 2% Na_2CO_3 in 0.1 M NaOH.

B 0.5% $CuSO_4.5H_2O$ in 1% sodium tartrate (separate double strength solutions mixed 1:1 before use).

C Folin-Ciocalteau reagent (Sigma) was diluted 1:1 with distilled water (to give a total acidity of exactly 1.0 N).

D 50 vol of reagent A was mixed with 1 vol of reagent B (renewed for each separate assay).

Procedure

Reagent D (5 ml) was added to sample (0.2 ml), containing 0 - 100 $\mu g\ ml^{-1}$ of protein, mixed well and left to stand at room temperature for 10 min. Reagent C (0.5 ml) was added rapidly with immediate mixing. Samples were left to stand for 30 min at room temperature then read against a reagent blank in a spectrophotometer at 750 nm with a light path of 1 cm. A standard curve was produced using bovine serum albumin.

C Phosphorus determination (Chen et al., 1956)

Reagents

1 Digestion mixture

conc. H_2SO_4:60% $HClO_3$ (3:2, v/v).

2 Colour reagent: 1 vol 6N H_2SO_4
2 vol distilled water
1 vol 2.5% ammonium molybdate
1 vol 10% ascorbic acid

Colour reagent was prepared fresh just before use.

3 Standard KH_2PO_4 solution (10 $\mu g\ ml^{-1}$).

Procedure

A few carborundum chips were added to samples (0.2 ml) containing 0 - 10 $\mu g\ ml^{-1}$ of phosphorus, in pyrex tubes. Digestion mixture (0.1 ml) was added to samples

which were treated at 140°C for 1 h in an oven. After cooling, water (3.9 ml) was added to samples followed by colour reagent (4.0 ml). Tubes were covered with nescofilm, mixed by inversion and incubated at 37°C for 1.5 h. Samples were read in a spectrophotometer (Schimadzu PR-1) at 820 nm against a reagent blank. A standard curve was produced using KH_2PO_4.

II PREPARATION OF STOCK SOLUTION FOR SDS-PAGE

(Laemmli method)

Separating and stacking gels contained 15% and 5% (w/v) acrylamide respectively. Gels and electrode buffers contained 0.1% (w/v) SDS. Gels were prepared in glass moulds and electrophoresis was done in a Pharmacia gel electrophoresis unit.

Stock solutions

1 Acrylamide (30.0 g) and NN'methylenebisacrylamide (0.8 g) was made up to 100 ml with distilled water. This was filtered and stored at 4°C.

2 1 M Tris-HCl buffer (pH 8.8) which was made up with 2 M Tris (50 ml), 1 N HCl (16.2 ml) and H_2O (33.8 ml). This was stored at 4°C.

3 0.5 M Tris-HCl buffer (pH 6.8) which was prepared by making up 1 M Tris (50 ml) and adding 1 N HCl (45 ml) and H_2O (up to 100 ml). This was stored at 4°C.

4 Tris-Glycine buffer (pH 8.3) was made up at 10 times the required concentration. This was prepared by adding glycine (144.2 g) and Tris (30.28 g) to 1 l of distilled water.

5 Ammonium persulphate 10% (w/v) was prepared the day before use.

6 SDS was made up at 20% (w/v).

7 Bromophenol blue was prepared at 0.1% (w/v).

8 Solubilizing buffer was made up containing the following stock solutions: 0.5 M Tris HCl pH 6.8 (25 ml), SDS (20 ml), β-mercaptoethanol (10 ml), glycerol (20 ml), bromophenol blue (2 ml) and distilled water (23 ml).

9 Fixing-staining solution was prepared containing Coomassie blue R250 (1.25 g), 50% methanol (454 ml) and glacial acetic acid (46 ml).

10 Destaining solution was made up containing methanol (50 ml), acetic acid (75 ml) and distilled water (875 ml).

11 Staining reagent for the silver stain was prepared by adding 20% silver nitrate (5 ml) to 0.1 M NaOH (28 ml) and concentrated NH_4OH (2 ml) while the solution was being mixed. Distilled water (115 ml) was added to give 150 ml of staining reagent.

III CALCULATION OF ADHERENCE INHIBITION INDEX (AII): WORKED EXAMPLE

Calculation of the AII, based on protein content, for the L-fucose bound fraction shown in Table 13 (2). The AII is a measure of the relative efficiency with which each component inhibits adherence as compared with crude EPM. At a concentration of 10 mg ml^{-1}, crude EPM from C. albicans GDH 2023 inhibits adherence of this strain to buccal cells by 47.8%. In calculating the AII, the weight of protein in each component required to inhibit adherence by 50% was determined.

For protein eluted from L-fucose column (Table 13, experiment 2) 43.8 μg protein inhibited adherence by 42.5%

$$\therefore \frac{43.8 \times 50}{42.5} = 51.5 \text{ } \mu g \text{ protein are required to inhibit adherence by 50\%.}$$

To calculate the AII value the weight of protein in the crude EPM sample is divided by this value

$$\therefore \quad AII = \frac{1}{51.5} \times 887.8 = 17.2$$

Therefore, the component eluted from the L-fucose column is 17.2 times more efficient than crude EPM from C. albicans GDH 2023 at inhibiting adherence of this strain to epithelial cells.

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