Effect of sub-inhibitory concentrations of antifungal drugs on adherence of Candida species

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EFFECT OF SUB-INHIBITORY CONCENTRATIONS OF ANTIFUNGAL DRUGS ON ADHERENCE OF CANDIDA SPECIES

BY

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A DOCTORAL THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY OF LOUGHBOROUGH UNIVERSITY OF TECHNOLOGY

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M.A. GHANNOUN, Ph.D.

DEPARTMENT OF CHEMISTRY, LOUGHBOROUGH UNIVERSITY, U.K.

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My sincere thanks go also to my wife Fatema Al-Haddad and children Salma and Hussein for their support, patience and forbearance.
TO

MY WIFE AND CHILDREN
SUMMARY

The adherence of three *Candida* species to human buccal epithelial cell (BECs) following treatment of the yeast with sub-inhibitory concentrations of amphotericin B, nystatin, miconazole nitrate, 5-fluorocytosine, octenidine and piritenidine was investigated *in vitro*. Pre-incubation of *C. albicans* (two strains), *C. tropicalis* or *C.kefyr* with these antifungal drugs inhibited their adherence to varying degrees (reduction between 17% and 78% of the control value). Pre-treatment of yeast for a short period (1h) had less effect on adhesion than pre-treatment for a long period (24h). Furthermore, treating *C. albicans* with a combination of amphotericin B plus 5-fluorocytosine, both at 1/8 MIC level, led to stronger adherence inhibition than that obtained for yeast pre-treated with either one alone at 1/4 MIC levels. In addition, the pre-treatment of either *Candida* or BECs or both types of cells with the drugs reduced adherence, the reduction being greatest when both types of cells were pre-treated. No difference in adherence between stationary or exponential phase yeast to BEC was observed and the drugs were effective in reducing the adherence of cells from either growth phase.

Exposure of *C. albicans* to antifungal drugs affected the outer cell envelope, as observed by scanning and transmission electron microscopy. It also suppressed germination and budding significantly, again to a different extent depending on the antifungal drugs used. Compared with the control-grown yeasts, an increase in the excretion of extracellular polymer into the supernatant of yeast cultured with various antifungals was observed. Chemical composition of this material showed that it is mannoprotein in nature containing hexoses (85-90%) and protein (7-9%). Mannose was the major sugar making about 87% of the total carbohydrates. Our results suggest that antifungals and two new antimycotic drugs, at sub-inhibitory concentrations, have multiple effects on *Candida* and point to the possibility of using these drugs in the prophylaxis against candidosis.
Murine intestinal disks and an infant mouse model were used to explore the effect saccharides have on the gastrointestinal (GI) colonisation and dissemination by C. albicans. With murine intestinal disks, inclusion of either mannose, N-acetylglucosamine (GLcNAc) or a chitin soluble extract (CSE), in the adhesion assay mixture in vitro, led to blockage of adherence of C. albicans to intestinal tissues to varying degrees with CSE being the most active. Pre-treatment of infant mice with these saccharides led to a significant reduction in GI colonisation and systemic spread of C. albicans from the gut to visceral organs. Results obtained following single dose, pre and post-treatment with saccharides suggest that dosing regimen employed for saccharides treatment is important.
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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>A°</td>
<td>Angstrom unit. A unit of length = 10^-10 metres</td>
</tr>
<tr>
<td>AMB</td>
<td>Amphotericin B</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BEC</td>
<td>Buccal Epithelial Cell</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CSE</td>
<td>Chitin Soluble Extract</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>EC</td>
<td>Epithelial Cell</td>
</tr>
<tr>
<td>5-FC</td>
<td>5-Fluorocytosine</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-Acetylglucosamine</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>Ig A</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>KCCC</td>
<td>Kuwait Cancer Control Centre</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MFC</td>
<td>Minimum Fungicidale Concentration</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>MN</td>
<td>Miconazole nitrate</td>
</tr>
<tr>
<td>NY</td>
<td>Nystatin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
</tr>
<tr>
<td>SDA</td>
<td>Sabouraud dextrose agar</td>
</tr>
<tr>
<td>SDB</td>
<td>Sabouraud dextrose broth</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>spp</td>
<td>Species</td>
</tr>
<tr>
<td>SPS</td>
<td>Sterile Physiological Saline</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>VEC</td>
<td>Vaginal Epithelial Cell</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>YNBG</td>
<td>Yeast nitrogen base plus glucose</td>
</tr>
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</table>
CHAPTER ONE

ANTIFUNGAL AGENTS AND THEIR MODE OF ACTION
Antifungal Agents and Their Mode of Action

There has been a marked increase in the number of systemic mycoses in recent years particularly in immunocompromised patients (1-3), surgical patients (4,5), artificial, internal prostheses, intravenous catheterisation and hyperalimentation patients (6-10). In addition to these infections, factors such as the use of cytotoxic chemotherapy may create ecological situations in which the risk of emergence of opportunistic fungal infections is increased (11-15). Many antifungal agents have been developed recently (16,17), but only a few are active both in vitro and in vivo. The most important antifungal agents which are clinically used for the treatment of candidosis can be divided into two major groups on the basis of their origin: [1] polyene antibiotics, which are produced by Streptomyces species, e.g. amphotericin B and nystatin (Table 1 & Figure 1); [2] synthetic agents, e.g. fluucytosine and N-substituted imidazoles (Figure 3).

A. Polyene Antibiotics

The discovery of nystatin by Hazen and Brown in 1950 (18,19) led to the isolation and characterisation of numerous antibiotics. These compounds share certain chemical and biological properties and are designated as the polyene antibiotics. Around 100 polyenes have been produced by a variety of filamentous bacteria, usually Streptomyces spp. and there is good reason to believe that there will be many more polyene antibiotics to be discovered (20).

Although the list of polyenes is impressive, problems associated with their stability, solubility, toxicity and absorption have made that only a few of these compounds are therapeutically useful. Table 1 lists those polyene antibiotics that have been shown activity against Candida. Of this number only a few are sufficiently non-toxic to be used clinically and only nystatin, amphotericin B, natamycin and candididin have found utility in the therapy of human fungal diseases (17).
Table 1. Polyene antifungals that have been shown to inhibit the growth of Candida species in vitro or in vivo.

<table>
<thead>
<tr>
<th>Name of Polyene Producing Organism</th>
<th>Chemical Composition</th>
<th>Mol. wt. (daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B: <em>Streptomyces nodosus</em></td>
<td>C_{47}H_{73}NO_{17}</td>
<td>924</td>
</tr>
<tr>
<td>Candidin: <em>S. griseus</em></td>
<td>C_{63}H_{85}N_{2}O_{19}</td>
<td>1,200</td>
</tr>
<tr>
<td>Candidin: <em>S. viridoflavus</em></td>
<td>C_{47}H_{71}NO_{17}</td>
<td>922</td>
</tr>
<tr>
<td>Etruscomycin (Lucensomycin): <em>S. lucensis</em></td>
<td>C_{36}H_{56}O_{14}N</td>
<td>739</td>
</tr>
<tr>
<td>Filipin complex: <em>S. filipensis</em></td>
<td>C_{35}H_{58}NO_{11}</td>
<td>655</td>
</tr>
<tr>
<td>Hamycin: <em>S. primprina</em></td>
<td>C_{57}H_{89}N_{2}O_{14}</td>
<td>1,147</td>
</tr>
<tr>
<td>Natamycin (Pimaricin): <em>S. nataliensis, S. chatanoogensis</em></td>
<td>C_{33}H_{47}NO_{14}</td>
<td>666</td>
</tr>
<tr>
<td>Nystatin: <em>S. albicus, S. noursei</em></td>
<td>C_{47}H_{75}NO_{17}</td>
<td>926</td>
</tr>
<tr>
<td>Trichomycin: <em>S. hachijoensis, S. abikoensis</em></td>
<td>C_{61}H_{88}N_{2}O_{21}</td>
<td>1,219</td>
</tr>
</tbody>
</table>
1. **Physical and Chemical Properties**

The UV spectra of all polyenes considered to be the most characteristic and useful physical property which have a regular series of sharp peaks of absorption, which are separated by sharp troughs all in the range of 280-400 nm (21). Norman *et al.* (22) related these UV absorption properties to the biological and biochemical mode of action of the polyene antibiotics.

Polyenes show limited solubility in both water or non-polar organic solvents such as ethers, alcohols or esters. In contrast to non-polar organic solvents, the polyenes are dissolved in polar organic solvents, such as dimethyl sulfoxide (DMSO) or dimethyl formamide (DMF). Stock solutions of polyene antibiotics in DMSO or DMF are stable if extreme care is taken to shield them from oxygen and light to which they are very sensitive.

Polyene antibiotics are characterised by a large macrolide ring of carbon atoms closed by formation of an internal ester or lactone (Figure 1). The macrolide ring contains 12-14 up to 35-37 carbon atoms and the conjugated double bond is contained exclusively within the cyclic lactone. Another highly characteristic feature which is common to all polyenes is the presence of a large number of hydroxyl groups, from 6-14 such groups. They are usually distributed along the macrolide ring on alternate carbon atoms. The presence of highly polar and non-polar regions within the molecule renders the polyenes amphipathic, accounting for their peculiar solubility properties. This amphipathic feature also plays an important role in the mode of action of these substances as they interact in various biological systems (20).

The acidity of these polyenes is due to a single carboxyl group, and the basicity to a single amino group. Some polyenes contain both a single amino group and a single carboxyl group and they are, therefore, zwitterions. The amino group present in some of the polyene antibiotics is associated with an amino sugar that is connected to the macrolide ring through a glycosidic
Figure 1. The structural formulae of certain clinically important polyene macrolide antibiotics.
bond. In all instances the carbohydrate moiety has been found to be mycosamine. Its elemental analysis identifies it as C$_6$H$_{15}$O$_4$N, and its structure as 3-amino-3,6-dideoxymannose (23). This is a 3-amino analogue of D-rhamnose or 6-deoxy-D-mannose. This mycosamine has been found in amphotericin B, candididin, nystatin, trichomycin, pimaricin, candidin and etruscomycin.

2. Mode of Action of Polyenes

In the early 1960's several laboratories independently presented evidence that polyene antibiotics could increase the cell membrane permeability of a number of organisms, promoting leakage of important cellular constituents and ultimately lysis and death of the cell (24,25). Many other studies have substantiated these findings and have reinforced the belief that other metabolic effects of the polyenes, such as inhibition of aerobic and anaerobic respiration observed in cells treated with polyenes, are a result of cytoplasmic leakage due to an alteration of cellular permeability and not due to inhibition of glycolysis or some other essential metabolic pathway (21,26). Since the plasma membrane is the structural unit of the cell responsible for the maintenance of selective permeability, it was reasonable that it should be considered the principle site of action of the polyenes. Inhibition of fungal growth by polyene antibiotics depends on the binding of the drug to the cell, and only cells that bind appreciable amounts of the polyene are sensitive. Bacteria, intact and as protoplasts, did not take up polyenes and therefore unaffected by polyenes. These results led to the hypothesis that the polyenes worked by interacting with membrane sterols which are major components of eukaryotic cell membranes, but not present in bacterial cell walls or membranes (20).

There is a direct association between the sensitivity of an organism to a polyene and the presence of sterols in the plasma membrane of the cells. All organisms susceptible to polyenes (e.g. yeasts, algae, protozoa and mammalian cells) contain sterols in their outer membranes, and all resistant organisms
do not contain sterols (27). Studies with *Mycoplasma laidlawii* (*Acholeplasma laidlawii*), an organism that is also unable to synthesize sterols *de novo*, provide further proof of the association between the sensitivity of an organism to a polyene and the presence of sterols (28, 29). When *M. laidlawii* cells were grown in a sterol-deficient medium, they were resistant to polyenes, but, if grown in sterol-containing media, sterols were incorporated into the plasma membrane and the organisms then become sensitive to polyenes (29).

The importance of membrane sterols for the action of polyenes is also supported by the finding of Gottleib *et al.* (30), Lampen *et al.* (31), Zygmunt and Tavormina (32). They showed that fungi can be protected from the inhibitory action of certain polyene antibiotics by the addition of sterols to the growth medium. This effect is due to a physico-chemical interaction between sterols added and the polyenes, which prevents the antibiotics from interacting with cellular sterols. Fatty acids also protect sensitive organisms against the action of polyene antibiotics (33), presumably through a similar mechanism. Sterols can also have the opposite effect. Mas and Pina (34) found that addition of ergosterol to cultures of nystatin-resistant strains of *C. albicans* which lack ergosterol, resulted in the organism becoming sensitive to the antibiotic. This was a phenotypic change, and on subculture in the absence of the sterol, these strains rapidly regained resistance.

The interaction between the sterols and polyene antibiotics are supported further by a direct evidence based on spectrophotometric data. Lampen *et al.* (31) reported that when sterols were added to aqueous solutions of the polyene antibiotic, filipin or nystatin, the UV absorbance values decreased significantly. This suggested that a direct interaction had occurred between the added sterol and the polyenes. Several other researchers have confirmed these findings (22, 27, 35, 36). Schroeder *et al.* (37) provided direct evidence for binding between sterols and filipin.
by using a fluorometric technique involving the measurement of partial quantum efficiency.

A correlation also exists between the degree of membrane damage caused by individual polyene antibiotic and the observed effects seen in electron micrographs. Freeze-etch electron microscopy demonstrated that polyene antibiotic filipin, but not amphotericin B, induces the formation of aggregates 15-25 nm in diameter (or "pits") in cholesterol containing membranes from A. laidlawii. These aggregates can not be considered as pores since no "through and through" holes were visible on the etched faces (38,39). Similarly, studies carried out on rat erythrocytes showed the same results as the work in A. laidlawii (38,40), although structural alterations [pits, doughnut-shaped craters and protrusions] in the erythrocyte/s membrane were apparent. For the larger polyenes (amphotericin B and nystatin) it has been proposed that the interaction of the antibiotic with membrane sterols results in the production of aqueous pores consisting of an annulus of eight amphotericin molecules linked hydrophobically to the membrane sterol (39,41). This gives rise to an aqueous pore in which the hydroxyl residues of the polyene face inwards to give an effective pore diameter of from 0.4 to 1.0 nm. The length of the annulus is such that two half pores are required to span the plasma membranes. The carbohydrate moiety of the polyene and the hydroxyl group of the membrane sterols are at one end of the complex located at the lipid-water interface, whereas at the other end of the complex within the lipid bilayer there is a single hydroxyl from the polyene molecule (42,43).

All the above evidence points to the fact that polyenes spectral changes totally depended on the presence of a sterol, whether it was free in solution, bound in a liposomal bilayer, or present in a naturally occurring membrane.

When comparable studies, with additional polyene antibiotics, were carried out, the results were similar and the
order of effectiveness of interaction with cholesterol was filipin > amphotericin B > etruscomycin > nystatin = natamycin (22,39,44-46). It was also observed that etruscomycin and amphotericin B, like filipin, were also capable of interacting with sterols bound to liposomes, to membrane bound sterols of red cell ghosts or to Acholeplasma membrane-bound cholesterol (39,44-46).

Other factors may also play a role in the cell sensitivity to polyenes. Hsu-chen and Feingold (47) reported that the presence of cholesterol in liposomes derived from egg lecithin were more sensitive than those derived from dipalmitoyl or distearoyl lecithins to nystatin or amphotericin B. Thus, it appears that polyene antibiotic toxicity may also be dependent on the fatty acid composition of the phospholipids. Again, the correlation between polyene sensitivity of different cell lines and the cholesterol:phospholipid molar ratio reflects a relation between sensitivity and the fluidity of the lipid in the surface membrane, and any changes in this ratio will affect the internal viscosity and molecular motion of lipids within membranes, and this may result in the differences in sensitivity of cells to amphotericin B. Because of all these variables, the mechanism's of selective toxicity of the polyene is a complex phenomenon.

B. Flucytosine

Flucytosine (5-fluorocytosine, 5-FC) is an oral antimycotic agent that has been synthesized by Duschinsky et al. in 1957 (48) during a search for cytostatic and antileukaemic agents. Although flucytosine lacks any such activity, it was investigated further on the basis of antifungal properties by Berger and Duschinsky (49) who demonstrated in vitro activity of this compound against yeasts. Grunberg et al. (50) examined its therapeutic activity in experimental candidosis and cryptococcosis in the mouse, and the first successful use of oral flucytosine to treat human candidosis and cryptococcosis was published in 1967 by Grunberg et al. (51).
1. **Antifungal Spectrum**

5-FC has a very marked selective antifungal activity in vitro and in vivo against pathogenic yeasts of the genera *Candida*, *Torulopsis*, *Cryptococcus* and *Geotrichum*, and moderate activity on fungi such as *Aspergillus* spp. The remaining fungi of medical importance such as *Coccidioides* spp., *Histoplasma* spp., and dermatophytes did not respond and all are resistant (52-54).

2. **Mode of Action**

Flucytosine exerts both fungicidal and fungistatic activity against yeasts (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. neoformans*), although the fungicidal effect requires relatively high concentrations and longer periods of exposure (53,55).

5-FC is actively transported into fungal cells by means of cytosine permease, which is usually responsible for the uptake of cytosine, adenine, guanine and hypoxanthine (56,57). Once inside the fungal cell, 5-FC is deaminated to 5-FU by a cytosine deaminase, an enzyme which is either absent or possesses low activity in animal cells (52) [Figure 2]. This circumstance is believed to be responsible for the differential toxicity between yeast and animal cells. The spectrum of activity of flucytosine is determined by the presence or absence of this enzyme. 5-FU by itself can not be used directly as an antifungal agent since its uptake, in low concentrations, into the fungal cells is poor, and at high concentrations, it is too toxic to mammalian cells (56). In *C. albicans*, 5-FU is subsequently anabolised to 5-fluoro uridine monophosphate by uridine monophosphate pyrophosphorylase (URMP) which is then incorporated into RNA resulting in the disruption of protein synthesis (58). Polak and Wain (59) suggested that in addition to incorporation of the deaminated drug into RNA, there may also be an independent inhibition of DNA synthesis. This is supported by Diasio *et al.* (58) by their finding that 5-fluorodeoxy uridine monophosphate (FUDRMP) which is present in cells of *C. albicans* treated with 5-FC produces a marked inhibition of thymidylate synthetase, a key enzyme in
the synthesis of DNA, which is consistent with blockage of DNA synthesis [Figure 2] (60). Wain and Polak (61) showed that 5-FU (from 5-FC) is ultimately incorporated in large quantities in the 80 S ribosomes of *C. albicans*, but the number of ribosomes synthesized in the high concentrations of 5-FC is greatly reduced as compared to non-treated cells.

Other consequences of the action of 5-FC on yeasts, particularly on *C. albicans* includes morphological and ultrastructural changes (62-64). The mean diameter of yeast form of *C. albicans* cells grown at sub-inhibitory concentrations of 5-FC increased in size, while the mycelial form continued to extend (62). This increase in size, in spite of inhibited DNA and RNA synthesis, was found to be due to continued or even excessive synthesis of carbohydrates and, to a lesser extent, proteins. Ultrastructural changes occur in both the nucleus and cell wall of *C. albicans*. After 2 hours of incubation with this drug, the nucleus become larger, while after 12 hours the nucleus is further enlarged and translucent with filamentous components appeared in it while the cell wall becomes progressively thinner (63). Yeast cells of *C. albicans* and *C. neoformans* showed more budding with 5-FC (65). Scanning electron microscopy of *C. albicans* yeast cells treated with 5-FC exhibited slightly folded surfaces as compared to smooth surfaces in un-treated cells (66).

C. Azole Antifungals

The first reports of the fungicidal properties of N-substituted imidazoles were made towards the ends of the 1960s by research groups at Bayer and Janssen (67). These original compounds have proved to be important drugs for combatting human fungal infections. The great success ofazole derivatives has prompted a continuing search for superior azole antifungal. Over 40 of the β-substituted 1-phenethyylimidazole derivatives were reported to have potent activity against yeast-like fungi, dermatophytes and also against Gram-positive bacteria (68,69).
Figure 2. Metabolic conversions and mode of action of 5-fluorocytosine in sensitive fungi (60).

5-FC = 5-fluorocytosine
5-FU = 5-fluorouracil
5-FUMP = 5-fluorouridine monophosphate
5-FUTP = 5-fluorouridine triphosphate
5-FdUMP = 5-fluorodeoxy uridylate
5-FdUDP = 5-fluorodeoxyuridine diphosphate
dUMP = deoxyuridylate
dTMP = deoxythymidylate
The imidazoles in current clinical use are: clotrimazole, miconazole, econazole and ketoconazole (Figure 3).

There are two common features in their chemical structures that are required for their activity (70). The first is possession of an unsubstituted imidazole and the second is a N-C covalent linkage between the imidazole and the rest of the molecule (Figure 3). The molecular weight of imidazoles range from 279 to 706 dalton (67).

1. Antimicrobial-Activity

Azole derivatives have a broad spectrum of activity in vitro. The growth of almost all pathogenic yeasts, particularly Candida spp., Cryptococcus spp. and Aspergillus spp., fungi of clinical interest (e.g. Blastomyces dermatitidis, Coccidioides immitis and Histoplasma capsulatum), and dermatophytes (e.g. Petriellidium boydii) are inhibited by various imidazole derivatives (67, 71, 72). Gram-positive cocci and bacilli of clinical interest (e.g. Staphylococcus aureus, Streptococcus pyogenes, Proteus spp., Salmonella spp.) show marked sensitivity towards various azole derivatives, but not against any genus of Gram-negative bacilli (67, 71-75).

2. Mode of Action of Imidazoles

Most of what is known about the mode of action of the imidazoles results from the chemical and electron microscopic investigations on C. albicans by Iwata and his co-workers in Japan (76, 77). Their studies revealed that the drug inhibited the synthesis of protein, RNA, DNA, lipid, mannan, and glucan. Further studies with cell-free system prepared from C. albicans showed that these drugs did not act directly on the mechanism of protein synthesis (76). In the same study, Iwata et al. (76) showed that at fungicidal (50 µM), but not fungistatic (13 µM), concentrations, clotrimazole caused massive release of intracellular phosphorus and potassium (labelled ³²P or ⁴²K) which occurred very soon after addition of the drug to grown cultures of C. albicans, and
Figure 3. Structural formulae of imidazoles used to treat fungal infections.
it was concluded that clotrimazole acts primarily on the cell membrane and that depletion of essential ions and metabolic precursors leads to inhibition of macromolecular synthesis. Similar results were obtained by Swamy et al. and Van den Bossche (78,79).

At a biochemical level, Van den Bossche et al. (80) showed that miconazole at low concentration could block the synthesis of ergosterol in C.albicans and that several 14α-methyl-sterols [24-methylene-dihydrolanosterol, lanosterol, obtusifoliol, 4,14-dimethyl-zymosterol, 14-methylfecosterol] accumulated in the cells (Figure 4). In addition, these workers showed that miconazole interfered with triglyceride synthesis in C.albicans, albeit to a lesser extent (80). This observation suggested that miconazole was a potent inhibitor of the 14α-demethylation step in fungal sterol biosynthesis. Similar findings of inhibition of 14α-demethylation by low concentrations of azoles was subsequently shown to be as common effect to all theazole compounds so far tested (81-84,96) (Figure 4). Other workers showed that the primary target of imidazole derivatives was the haem-protein that co-catalyses 14α-demethylation of lanosterol - cytochrome P-450 (17). The differences in the extent of inhibition of fungal and mammalian cytochrome P-450s account for the selective toxicity of azoles for fungi (85-87).

Further evidence support the interactions between the imidazole antimycotics and plasma membrane was presented by Yamaguchi (88,89). He showed that the antifungal activity of both clotrimazole and miconazole is antagonised by several classes of lipids containing unsaturated fatty acids. Similar results has been reported by Van den Bossche et al. with ketoconazole (90). The drugs may alter the organisation of membrane lipids without necessarily binding to them (90). In addition to its direct action on plasma membrane, miconazole and ketoconazole have been shown to inhibit the ATPase of cell membrane of C.albicans and other yeasts (91,92). Whether or not this effect accounts for
Figure 4. Inhibition of sterol demethylation byazole compounds (95).
the rapid collapse of electrochemical gradients (91) and fall in intracellular ATP (93) is not known. Such action could result from the uncoupling effects of membrane lipid interactions of azoles as well as from enzymic inhibition of the membrane ATPase (17). Surarit and Shepherd (94) reported that miconazole and ketoconazole at growth inhibitory concentrations extensively inhibit the C. albicans plasma membrane enzyme ATPase, glucan synthase, adenylcyclase and 5-nucleotidase, when assayed in situ (94). In the same study, they reported similar inhibition of plasma membrane enzymes with polyenes (nystatin and amphotericin B).
CHAPTER TWO

STRUCTURE AND COMPOSITION OF CANDIDA CELL ENVELOPE
**Candida albicans** is a dimorphic yeast which is capable of growing in culture as an ellipsoidal bud (usually referred to as a blastospore or yeast form), as a pseudohypha of elongated yeast like cells or as a true septate (referred to as filamentous, or hyphal form), depending on the environmental conditions and the growth history of the cells (17,97). In infected tissues, both forms are invariably found, but it seems likely that the hyphal form plays a pathogenic role in the initial processes of tissue invasion (17). The cell wall of *Candida* is of critical importance for several reasons: it forms the contact point for adhesion of the fungus to host cells; it maintains the shape of the cell; it acts as a barrier to drugs (98); it contains components that act as immunogenic determinants and it is intimately involved in the secretion of hydrolytic enzymes (99).

*Candida* cells adhere through their walls whereas epithelial cells [ECs] do so through their plasma membranes. Therefore, an understanding of the chemical composition making up the surface envelopes of *Candida* as well as EC is an essential pre-requisite for understanding the molecular bases of the adhesion process.

I. CANDIDA CELL WALL

1. Chemical Composition of Candida Cell Wall

Yeast walls are composed mainly of two polysaccharides, α- mannan and β- glucan, making about 75-85% of the dry matter, with minor amounts of chitin, protein and lipids (100 -104). The cell wall of *C.albicans* comprises some 30% of the dry weight of the cell and is composed predominantly of mannoproteins (20-30%) and β- glucans (50-60%) with small quantities of chitin (0.6-2.7%), protein (3 -6%) and lipid (2%) (105). Total carbohydrate content in whole cells and cell walls of *C.albicans* have been found to be similar in the two morphological forms (101,106), although differences in glucan and mannan content have been noted in fractions of cell walls and whole cells from the two forms (101,102). Chattaway et al. (101) reported that an alkali-insoluble fraction from mycelial walls contained 3 times more chitin than
those of yeast cells which is consistent with the findings of Sullivan and his co-workers (105). Differences in amino acid composition of cell wall proteins as well as lipid content was noted between the two morphological forms (101,102, 107, 108).

A: Glucan

Glucans and mannans, are frequently combined with proteins in cell wall extracts as glycoproteins, mannoproteins and glycomannoproteins (109). Such substances are believed to originate in the cell walls of *Candida* since similar molecules are often detected in culture metabolites (109,110). The term glucan is applied to a group of β-glucose polymers linked by β-glycosidic linkages. Although α-glucans have been found in the walls of a small group of filamentous fungi but not detected in yeast (111). In most cases glucans of fungal walls contain mixed bonds, and in yeast walls these bonds are predominantly of the types β -(1-3) and β- (1-6) (112). Glucan appears to be the most abundant polymer in the cell wall of *C.albicans*. It consists of a backbone of β-1,6- linked glucose residues to which side chains of β-1,3-linked glucose residues are attached (103,113-115). Bishop et al. (113) showed that the glucan extracted with boiling NaOH comprised about 70% of β -(1-6) linked and terminal residues. An analysis of the acid-soluble and the insoluble glucan fractions from yeast, germ-tube, and hyphal cells of *C.albicans* showed that the acid-soluble fraction of each cell type consisted mainly (70%) of β -(1-6)-linked glucose residues and 5-8% of branch-point residues, while in the alkali-insoluble glucan, the relative amounts of β-(1-3) and β-(1-6) linkages were 30% and 50% from yeast and mycelial cells, respectively (114). Interestingly, β-(1-3) linkages accounted for 67% of the residues in the alkali-insoluble glucan from germ-tube forming cells and only 14% β-1,6 linkages (114). Shepherd et al. (115) proposed that at least three distinct glucans existed in *C.albicans* wall:[1] an alkali-soluble polymer of relatively low molecular weight,[2] a branched acid-soluble glucan containing mainly β -(1-6)-linked residues and [3] an insoluble highly branched complex with variable amounts of β -1,3- and β -1,6-linkages (Figure 5). Part of the insoluble glucan is linked to chitin (115).Yu et al.(103) and Bishop et al.(113) gave a detailed chemical study of alkali-
Figure 5. Structure of $\beta$-glucans in the cell wall of \textit{C. albicans}. A highly branched $\beta$ 1,6- glucan, $\Delta$; a highly branched $\beta$ 1,3- glucan, $\Box$; and a mixed $\beta$ 1,3/ 1,6- glucan complexed with chitin, $\bigcirc$. 
soluble glucan preparations from *C. albicans* serotype A and B, and from *C. parapsilosis*. All of the glucan preparations were of relatively low molecular weight, were highly branched, and contained a high proportion of β-(1-6) linked residues. The glucan from *C. albicans* serotype A differs from the others in having a higher degree of branching and few, if any, β-(1-3) linked glucose residues (103, 113).

Gale et al. (116) showed that stationary phase cultures of *C. albicans* were more resistant to polyene antibiotics, such as AMB, than exponentially grown cells. This resistance is associated with the cell wall. Protoplasts of stationary phase organisms have the same sensitivity to AMB as compared to those from exponentially grown organisms; the difference in sensitivity of the cells must therefore lie in alterations in the cell wall (116). Cytochemical studies have shown the presence of β-(1-6)-glucan and mannan at the outer surface of the cell walls, and in the case of exponentially grown organisms, there was less staining in the inner regions where β-(1-3)-glucan and chitin are believed to be more abundant (98). By contrast, walls from stationary phase cultures gave a more uniform staining reaction, indicating a greater intermixing of the four polysaccharide components (98). Treatment of stationary phase yeast cells with various hydrolytic enzymes, particularly an exo-(1-3)-β-D-glucanase, caused a decrease in the resistance to AMB, suggesting that the β-(1-3)-glucan could play a major role in the phenotypic resistance (117). Chitinase, trypsin, lipase, and α-mannosidase showed similar but less pronounced effects (116). Molina et al. (118) showed that a significant decrease in exo-1,3-β-glucanase upon the yeast-to-mycelium transition, indicating that the enzyme may not be necessary for mycelial growth.

**B: Chitin**

Chitin is a linear polysaccharide composed of N-acetylglucosamine (GlcNAc) [Figure 6] units joined by β-(1-4)-linkages, which is an important component of the cell wall of many filamentous fungi (101, 119). Chitin in *S. cerevisiae* has been shown to be associated with bud scars (90%) and the remaining
Figure 6. Structure of N-Acetyl-D-glucosamine.
10% being dispersed around the entire cell (120). In *C. albicans*, the majority of the chitin is located in the bud scars, but there is a layer of chitin close to the membrane and small portions distributed throughout the wall (99,106,121). Chitin chains are usually found as lattice of highly crystalline polymer within an amorphous polysaccharide or protein matrix.

Several studies have demonstrated that the formation of new walls in fungal protoplasts [*Saccharomyces*, *Aspergillus* and *Candida*] is initiated by deposition of a chitin skeleton which allows incorporation of other wall constituents (122). Sentandreu *et al.* (123) used the simile of chitin being the foundation of a building, responsible for the maintenance of the whole structure, and they hypothesize that the first step in wall formation is to build ground for the wall, i.e. form chitinous network on the cell surface. Glucans and high molecular weight mannoproteins are then deposited giving rise to complexes which might produce definite frameworks resulting in cell wall (123), although chitin helps to anchor glucan to the cell membrane, it is covalently linked to glucans (124). Wessels and co-worker (125,126) have shown that a considerable fraction of the β-glucan in the basidiomycete *Schizophyllum commune* is initially water-soluble, but becomes water and alkali insoluble after covalently linking to chitin. In *S. cerevisiae* a minor fraction (about 15%) of the total glucan might also interact covalently with chitin (127). It is speculated that in *C. albicans* there occurs a progressive cross linking between α-glucan and chitin during cell extension, leading to a change in the solubility characteristics of the glucan (124). However, the formation of the initial chitin network by protoplasts of *C. albicans* is independent of the other wall polymers; the mannoprotein incorporation occurs only in a coordinated way with the synthesis of β-glucan (124). Gow and Gooday (128) showed that chitin extracted from *C. albicans* was α-chitin (i.e. a polysaccharide chain folded in an antiparallel conformation) and is of the short fibril type. Chattaway *et al.* (101) compared the cell wall of *C. albicans* yeast and mycelial forms and reported that mycelial walls contained 3
times more chitin than those of yeast forms. This finding was subsequently supported by the observation that homogenates from *C. albicans* mycelial cells contained 2-fold more chitin synthetase activity than those obtained from yeast cells (105,106). This enzyme was present in a zymogenic state in both morphological forms. The synthetic pathway to chitin from glucose in *C. albicans* is illustrated in scheme 1 (129).

C: Mannoproteins

These polysaccharide-protein complexes, in which polysaccharides are covalently linked to proteins, represent the major antigenic component of intact yeast cells and accounts for about 20 to 30% of the wall by weight (130). Lectin binding, extraction of cells with different solvents, scanning and transmission electron microscopy, and labelling with ferritin-conjugated antibodies have shown that wall mannoprotein in *C. albicans* is distributed throughout the wall but is also accumulated on the surface and next to the plasma membrane (131-133). The results of Elorza et al. (99,124) studies on protoplasts of *C. albicans* indicated that walls are formed in two steps; the first involving a chitin microfibrillar skeleton formation and the second; the addition of glucan-mannoprotein complexes. Miragall et al. (134) suggested that chitin fibrils represent the fundamental component of the inner layers at early stages of wall regeneration, while mannoproteins are predominant in the layers of the cell wall.

The chemical composition of mannoproteins and their biosynthesis in various yeasts involve two types which are commonly differentiated: [1] the non-enzymatic mannoproteins directly involved in the architecture of the wall as building blocks, and [2] the enzymatic mannoproteins mainly contributed to the degradation of molecules to facilitate their uptake or to participate in the turnover of envelope components (130).

i. Structure of Non-Enzymatic Mannoproteins

Mannan is a generic name for different polysaccharides having in common a high proportion of mannose residues. The structure of *S. cerevisiae* mannan has been studied by several
Enzymes:

1. N-acetylglucosamine kinase
2. N-acetylglucosamine-6-phosphate deacetylase
3. Glucosamine-6-phosphate deaminase
4. Glutamine-fructose-6-phosphate aminotransferase
5. Glucosamine-phosphate acetyltransferase
6. Glucosamine-phosphate isomerase
7. UDP-acetylglucosamine pyrophosphorylase
8. Chitin synthase
Scheme 1. Pathway for chitin synthesis [From Shepherd et al. (1980) (129)]
researchers (100, 135,137). Mannans are usually covalently linked to protein, and apparently their role in yeasts is to hold together the different components of the yeast cell wall (106). Sentandreu and co-workers (124,138) showed that changes in a single sugar residue affected the overall conformation of a macromolecule, and the carbohydrate moiety of glycoconjugates changes during development, differentiation and transformation. The carbohydrate moiety can be attached to protein either by N-glycosidic links via GLcNAc unit to an asparagine residue in the peptide, or by O-glycosidic links to the hydroxy amino acids serine and threonine (139,140) [Figure 7]. Chemical analysis of S.cerevisiae mannans showed that they contain essentially two types of carbohydrate moieties differing in structure, size, and mode of attachment to protein (100,140). The first carbohydrate moiety of yeast mannan contains up to 150 mannosyl units and has a comb-like structure where the backbone is made of α-1,6-linked mannosyl units to which short chains of α-1,2- and α-1,3- linked mannooligosaccharides are attached predominantly by α-1,2-mannosidic bonds. A branched "inner core" region comprising 12 to 17 mannosyl units links the polysaccharide moiety to an asparagine residue in the peptide via an N,N'-diacetylchitobiose bridge. Some phosphate was found in the outer region of the mannan molecule serving as phosphodiester bridge in the side chains [Figure 7] (141). The differences between the inner core and the outer chain regions are [1] the existence of phosphomannose residues in the outer chain, and [2] the presence, in the inner core, of mannose units joined with α-(1-3) bonds to the α-(1-6) linked polymannose backbone (142).

The second carbohydrate moiety of yeast mannan consists of short mannooligosaccharides (up to mannottetraose) containing both α-1,2- and α-1,3-glycosidic bonds and attached at their reducing end by O-glycosidic links to serine and/or threonine in the peptide (143).

Mannans derived from the cell walls of C.albicans contain predominantly α-1,6 and α-1,2 linked mannopyranose residues. They commonly contain less phosphorous than the mannans of S.cerevisiae (104,113). A very few α-1,3 bonds have also
Figure 7. Structure of N-glycosidically linked (A) and O-glycosidically linked (B) mannan in *S. cerevisiae*. [M] mannan; [GNAc] N-acetylglucosamine (137).
been recorded between the mannose units (114). There is a polysaccharide with \( \alpha-1,6 \) mannan backbone with side chains containing up to 6 hexose monomers, linked by \( \alpha-1,2 \) and a few \( \alpha-1,3 \) bonds. Variations between mannans of serotypes A and B of \( C. albicans \) and \( C. stellatoidea \) are related to the extent of branching (104), the side chain length and the frequency of \( \alpha-1,3 \) bonds in the side chains. There appears to be a common similarity in the glucans and mannans from cell walls of various \( Candida \) spp. (115,142). Kogan et al. (145) carried out a comparative study of three cell-wall mannans of \( C. albicans \) serotypes A and B and \( C. parapsilosis \). They reported yeast mannans as having an \( \alpha-1,6 \)-linked backbone with some units being substituted at 0-2 with oligosaccharides joined by \( \alpha-1,2 \) and, to lesser extent, by \( \alpha-1,3 \) glycosidic bonds. Branching points in the side chains of \( C. albicans \) mannans were found in substantial proportions for the first time (145). Another study compared the wall mannoproteins of the yeast and mycelial cells of \( C. albicans \) (146). Zymolyase released between 20 and 25\% of the total protein from purified walls of the two forms of \( C. albicans \). The material released contained 492\% carbohydrate (86\% mannose and 6\% glucose) and 7\% protein (146). A great majority (86-88\%) of the carbohydrate was N-glycosidically linked to the protein with the remaining linked O-glycosidically (123,146).

ii. Structure of the Enzyme Mannoproteins:

Other mannose-containing proteins have been found in the periplasmic space of yeast cells. They seem to have an important role in cell morphogenesis [\( \beta \)-glucanases] or in cell economy [e.g. acid phosphates, invertase] (130). The presence of invertase on the outer surface of yeast cell was gained from investigations which showed that \( S. cerevisiae \) cells lost a large percentage of activity when they were converted into protoplasts, suggesting that the enzyme was located externally to the permeability barrier. Gascon and Ottolenghi (147) found two isoenzymes, one soluble in the cell sap, and with no mannose at all (small invertase), and the second one outside the plasmalemma and with a significant amount of mannan (large invertase). External invertase contains 50\% mannose and 3\% glucosamine, with a
molecular weight of 270,000 daltons (147,148). Acid phosphatase with a molecular weight of about 290,000 daltons also contains 40-50% mannose and 4% glucosamine, and seems to be variable with respect to its carbohydrate content (149). Scheme 2 summarises the possible pathways involved in the biosynthesis of the outer region of mannoproteins in \textit{S.cerevisiae} (130).

D. Lipids:

Lipids represent the last constituent of \textit{Candida} walls, and it makeup 1-5% of the dry weight and that the lipid content increases during the yeast-mycelial transformation (101). Many reports have appeared on the cell wall lipids of \textit{S.cerevisiae} and \textit{C.albicans} (107,108,150,151). Bianchi (107) found twice as much lipid in hyphae as in blastospore, and three times as much carbohydrate has been found in hyphal membranes as in yeast membranes. Ghannoum et al. (151) isolated a membrane-free preparation of \textit{C.albicans} cell walls and they found that lipids consisted of almost equal proportions of apolar and polar compounds. The major lipids found in the wall preparation were triglycerides , phospholipids, sterols and sterylester (151). The work of Suomalainen and Nurminen (150) and Ghannoum et al. (151) presents an alternative view point to the view held that cell walls of most yeasts are devoid of lipids.

2. Cell Wall Architecture

Numerous cytochemical and cytological studies showed that layering of the cell wall components is one of the most characteristic ultrastructural features of fungal cell walls (132,140,152,153). Interactions between various components making up the cell wall determine the way in which these component molecules are located inthe microbial cell and confer rigidity to this structure. The general picture is that the skeletal, microfibrillar wall components, such as \(\beta\)-glucan, chitin, and/or cellulose, are embedded in an amorphous polysaccharide and protein-polysaccharide matrix. The outer surface of the wall is usually smooth or slightly reticular, whereas the skeletal polysaccharide microfibrils are more prominent on the inner
Scheme 2. Schematic representation of the pathways leading to the synthesis of the outer region of mannoprotein in *S. cerevisiae* (130).

Dol = dolichol  
P = phosphate  
UDP = uridine diphosphate  
GLcNAc = N-acetylglucosamine  
UMP = uridine monophosphate  
Man = mannose  
Glc = glucose  
Prot = protein  
Asn = asparagine
surface of the wall (140). Sentandreu et al. (152) reported that both covalent and non-covalent interactions are present in the yeast cell wall. Covalent bondings exist between both chitin and glucan and mannan and glucan (152). Disulphide links, S-S, also play a role in holding together different mannoprotein moieties which lie on or near the cell surface (140). The cross-linking forms a permeability barrier against the escape of wall-located enzymes, as well as against the penetration of extra-cellular glucanases to the internal wall layer made of β-1,3 and β-1,6 glucan (140).

Non-covalent interactions in C.albicans and S.cerevisiae include ionic and cationic, hydrogen bonds interactions (140,152,154). Sentandreu et al. (152) speculated that differences in the cell wall of the two morphological forms of C.albicans, i.e. yeast and hyphae, could partially be accounted for by differences in the nature of interactions existing in their cell wall mannoproteins.

3. Cell wall Ultrastructure

Candida cell wall fulfills several functions connected with the interactions of the cell with its environment. Adherence of the fungal cell to the oral mucosal cells involved intimate contact between the EC surface and the deeper layers of the fungal cell wall (155). Several cell wall layers have been identified for C.albicans (156). Using the polysaccharide detection technique, Poulain et al. (153) suggested that eight constitutive layers (Lo to L8) are present in the cell wall of the blastospore of C.albicans. Cytochemical staining revealed that each layer contains a dominant polysaccharide (153,157). The gross appearance of the cell wall as viewed by electron microscopy showed that it comprises an outermost layer of high electron-dense, mostly mannoprotein, an inner layer of high electron-dense, mostly mannan, and an intermediate set of layers of lower electron-dense, chitin and glucan with some mannan (153,157,158) [Figure 8]. The overall cell wall thickness is approximately 250-260 nm. Many workers have reported that the layered structure varies with the cell age, growth environment
of the cells studied and probably arise from different fixative and staining techniques employed (153,156-159). Poulain and his colleagues (153) have shown that both the growth medium and the age of the cells affected the number and appearance of layers in the cell wall. Based on immunoelectron microscopic observations, Takamiya et al. (160) proposed a modification to the Poulain et al. scheme of the cell wall of C.albicans blastospores and suggested the addition of channels for mannan transportation [Figure 8] (160).

II. PLASMA MEMBRANE

A. Host Plasma Membrane

Every living cell is enclosed by a membrane, called the cytoplasmic membrane, that serves not only as a sturdy envelope inside which the cell can function but also as a discriminating portal, enabling nutrients and other essential agents to enter and waste products to leave. The plasma membrane also serves as receiver and transmitter of information enabling the metabolism of the cell to respond to its environments or extracellular factors (161). The cell surface is also the primary site for control of cell-cell interactions, cell division, development, growth and death.

Membranes are composed almost entirely of two classes of molecules: lipids and protein. The relative amounts of each of these classes of molecules vary considerably. The proteins are embedded in the lipid bilayer and serve as enzymes, or biological catalysts, and provide the membrane with its distinctive functional properties. The lipids provide the gross structural properties of the membrane (162). The cell membrane also contains about 5% carbohydrate, present as glycoproteins and glycolipids. Some of these constituents are found in the surface coat or the "glycocalyx" that surrounds many cell membranes (163). These components may be considered as part of the membrane structure, since they are firmly bound to the cell membranes (161).
Figure 8. Scheme of the *C. albicans* cell wall architecture modified from Poulain et al. (153) with suggested channels of mannan transportation [arrow] (160).

1 = mannan, glucans; 2 = mannan, glucans; 3 = mannan, glucans; 4 = mannan, glucans; 5 = glucans, chitin; 6 = mannan, ?, proteins; 7 = chitin, glucans; 8 = chitin, proteins, polysaccharides.
The most commonly accepted model of membrane structure is the so-called fluid - mosaic model (Figure 9). According to this model, the plasma membrane consists of a bimolecular lipid layer whose surface is interrupted by protein globules (164). The membrane proteins have been grouped into two classes (164). One is referred to as the integral (intrinsic) membrane protein. These proteins are firmly entrenched in the lipid bilayer, and are solubilized only with the aid of membrane-disrupting agents such as detergents and lipid solvents. The other class of membrane proteins is referred to as the peripheral (extrinsic) proteins, which localized predominantly or completely outside the lipid membrane framework (161). The membrane proteins can also be distinguished by a difference in their carbohydrate content. The membrane proteins can be further classified as either non-glycoconjugated proteins or glycoproteins. The carbohydrate residues found on glycoproteins are exposed only to the external surface of the plasma membrane (165). The cell surface carbohydrates have a role in cell adhesion, cell-cell interaction, and receptor function-processes which are involved in external communication.

Animal cell membranes contain three classes of lipids: phospholipids, sterols, and glycolipids (166). There are two types of phospholipids: glycerophosphatides and sphingophospholipids. The glycerophosphatides are the most prevalent among the membrane lipids. The major glycerophosphatides found in cell membranes are phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol (Table 2). Plasmalogens (containing an \( \alpha, \beta \)-unsaturated hydrocarbon residue ether-linked to carbon one of glycerol in place of the fatty acyl residue), lysophosphatides (containing only one fatty acyl residue), phosphatidic acid, and cardiolipin are also occasionally found in cell membranes as minor components (167). The sphingophospholipid, sphingomyelin, contains the basic structure sphingosine, to which a fatty acid is amide-linked to the amino group and a phosphorylcholine is estrified at the carbon one position (161). Cholesterol is the predominant neutral lipid of the plasma membranes. Other neutral lipids such as glycerides and cholesteryl esters are present as minor components (168). Plasma membranes have a characteristically
Figure 9. The fluid-mosaic model of membrane structure [From Hakomori, 1986 (173)].
### Table 2

**Lipids in Biological Membranes**

#### Glycerophospholipids

<table>
<thead>
<tr>
<th>Lipid</th>
<th>R</th>
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<tbody>
<tr>
<td>Phosphatidic acid</td>
<td>H</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>CH₂CH₂N⁺(CH₃)₃</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>CH₂CH(NH₂)COOH</td>
</tr>
<tr>
<td>Phosphatidylylycerol</td>
<td>CH₂CH(OH)CH₂OH</td>
</tr>
</tbody>
</table>

#### Sphingolipids

<table>
<thead>
<tr>
<th>Lipid</th>
<th>R</th>
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</thead>
<tbody>
<tr>
<td>Ceramide</td>
<td>H</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>PO₃CH₂CH₂N⁺(CH₃)₃</td>
</tr>
<tr>
<td>Glycosphingolipids</td>
<td>saccharides</td>
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</tbody>
</table>

#### Sterols

<table>
<thead>
<tr>
<th>Lipid</th>
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<tbody>
<tr>
<td>Cholesterol</td>
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</table>
high cholesterol to phospholipid ratio as compared to intracellular membranes.

Glycosphingolipids and glycoglycerolipids are the two major glycolipids found in mammalian cell membranes. Glycosphingolipids are composed of sphingosine, fatty acid, and carbohydrate, whereas glycoglycerolipids contain glycerol, fatty acid or fatty ether, and carbohydrate. Glycosphingolipids are present in most, if not all, animal tissues, while glycoglycerolipids have a narrower distribution (169). The constituent fatty acids of membrane phospholipids are normally a mixture of saturated and unsaturated with up to 5 double bonds or more per molecule. Membrane fluidity is strongly dependent on the composition of membrane lipids. A determinant of membrane fluidity is the degree of unsaturation and the length of the phospholipid acyl chains (170). Membranes containing mainly fully saturated long-chain phospholipids are more rigid and highly ordered. The plasma membrane also shows some degree of asymmetry, as far as the lipid composition is concerned. The type of such asymmetry varies from one membrane to another (171).

Polysaccharides are either attached to the peripheral protein globules or to polar groups of the phospholipids forming glycoproteins and glycolipids respectively (172). Each polysaccharide chain is relatively short and may branch. The constituent monosaccharides in membrane glycoproteins and glycolipids include D-galactose, D-mannose, L-fucose, N-acetyl neuraminic acid, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. In view of their hydrophilic nature they are frequently assumed to affect the orientation of the protein and lipid molecules, to which they are attached, toward the aqueous phase outside the cell (161). The membrane oligosaccharides are believed to be the agent through which cells of an organism can recognise foreign cells, thus triggering the immune response of the organism. Recognition is possible because the foreign cell membrane glycoproteins contain different carbohydrate markers than those present in their own membrane.
Among cells of the same tissue it is generally assumed that the surface polysaccharides act as an adhesive, linking these cells together. Oligosaccharides of the glycosphingolipids are believed to play a role in cell-to-cell recognition. Furthermore they are probably involved in the transmembrane transport and in providing the antigenic properties of the membrane (173).

B. Candida plasma membrane

The plasmalemma of Candida spp. is significant both as a prime target for attack by antifungal agents and as the location of enzymes involved in cell wall synthesis (17). The chemical analysis of the plasma membrane of yeast and mycelial forms of C.albicans showed that small amounts of nucleic acid (0.3 to 7%) and larger amounts of protein (25 to 65%), lipid (2.3 to 45%) and carbohydrates (3.2 to 30.8%) on dry weight bases were found (174). Cytoplasmic membrane from the yeast and mycelial forms of C.albicans showed marked differences in their phospholipids, free and esterified fatty acids, sterols, and total fatty acids, and the chemical composition varied with the growth phase (174). The major phospholipids present in the plasma membrane of C.albicans are phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, phosphatidylserine, and sphingolipid (174) [Table 2]. The last two phospholipids were present in membranes from yeast forms but not from mycelial forms. Membranes from yeast forms were much richer in free sterols and steryl esters, while mycelial forms were much richer in triglycerides and free fatty acids. The predominant sterol in plasma membrane of C.albicans was ergosterol, irrespective of the morphological form (174). Ghannoum et al. (108) confirmed that mycelial forms of C.albicans were more active than yeast forms in the accumulation of steryl esters and triacylglycerols. Furthermore, it was shown that yeast lipids contained much larger proportions of free sterols than the mycelial lipids, whereas the mycelial lipids contained larger amounts of sterols bound as steryl glycosides, predominantly cholesterylmannoside, than the yeast forms (108).

Acyl lipids from membranes of yeast forms contained higher proportions of linoleic acid (18:2) than those from membranes of
mycelial forms (108). Alternatively, apolar and polar lipid frac­tions from whole mycelial forms were found to contain higher levels of linoleic and linolenic acids (18:2 and 18:3) but lower levels of oleic acids (18:1) than the corresponding fractions from whole yeast forms (108). These differences could be of significance as far as the adherability is concerned.
CHAPTER THREE

PARAMETERS AFFECTING ADHESION OF CANDIDA
Parameters Affecting Adhesion Of Candida

The adherence of microorganisms to epithelial cell (EC) surfaces is now recognised as an important first step in the colonisation and invasion of mammalian tissues (175-178). During the last decade extensive investigations have resulted in the elucidation of mechanisms that mediate bacterial adherence to host cells (175), relatively few research efforts have focused on yeast adhesion. The adhesion of the pathogen, *C. albicans*, to any surfaces (living or inanimate) may vary considerably depending on yeast factors, EC factors, environmental factors and others. Douglas (176) categorised factors that affect the adhesion of Candida spp. to ECs in vitro to three comparable categories which include yeast, the ECs and the assay environment factors. Table 3 summarises various factors affecting the adhesion of *C. albicans* to mucosal cells and inanimate surfaces in vivo and in vitro (178).

1. YEAST FACTORS

a. Species and Strains

There are 196 spp. of *Candida* including *Torulopsis*, however only 15 spp. of *Candida* have so far been identified as facultative pathogenic (1,2,17,26). *C. albicans* is undoubtedly the most virulent spp., followed by *C. tropicalis* (26,179). These differences in virulence are closely paralleled by differences in the ability of the organisms to adhere to ECs in vitro (178,179). King et al. (180) examined the adherence capabilities of seven *Candida* spp. in vitro and showed that *C. albicans* adhered to vaginal and buccal epithelial cells (VEC and BEC) to a significantly greater degree than the other spp. tested. *C. tropicalis* and *C. stellatoidea* showed significant adherence to mucosal cells while *C. parapsilosis* adhered only to a slight degree. *C. guilliermondii*, *C. krusei* and *C. kefyr* [formerly *C. pseudotropicalis*] failed to interact with collected ECs (180).
Table 3. Factors affecting the adherence of *Candida* spp. to epithelial cells and to inanimate surfaces.

<table>
<thead>
<tr>
<th>Category</th>
<th>Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast factors:</td>
<td>(a) Species, strains, and phenotypes.</td>
</tr>
<tr>
<td></td>
<td>(b) Culture concentration.</td>
</tr>
<tr>
<td></td>
<td>(c) Culture age and viability.</td>
</tr>
<tr>
<td></td>
<td>(d) Culture medium.</td>
</tr>
<tr>
<td></td>
<td>(e) Germ-tube formation.</td>
</tr>
<tr>
<td></td>
<td>(f) Cell surface hydrophobicity.</td>
</tr>
<tr>
<td>Epithelial cell factors:</td>
<td>(a) Cell type.</td>
</tr>
<tr>
<td></td>
<td>(b) Viability and variability.</td>
</tr>
<tr>
<td>Environmental factors:</td>
<td>(a) Temperature and time of contact.</td>
</tr>
<tr>
<td></td>
<td>(b) Hydrogen-ion concentration and carbon dioxide level</td>
</tr>
<tr>
<td></td>
<td>(c) Saliva and serum.</td>
</tr>
<tr>
<td></td>
<td>(d) Hormonal status.</td>
</tr>
<tr>
<td></td>
<td>(e) Buffer used.</td>
</tr>
<tr>
<td></td>
<td>(f) Commensal bacteria.</td>
</tr>
<tr>
<td></td>
<td>(g) Antibodies.</td>
</tr>
<tr>
<td>Inanimate surfaces factors:</td>
<td>(a) Hydrophobic forces (London-Van der Waals forces).</td>
</tr>
<tr>
<td></td>
<td>(b) Surface free energy.</td>
</tr>
<tr>
<td></td>
<td>(c) Electrostatic forces.</td>
</tr>
<tr>
<td></td>
<td>(d) Type of surfaces.</td>
</tr>
</tbody>
</table>
Thus there appears to be a relationship between the ability of different spp. to adhere and their propensity for causing infection. Similar spp. differences have been reported for adhesion to epidermal corneocyte (181), vascular endothelium (182), fibrin-platelet matrices (183) and intravenous catheters (184) in vitro. These differences in adherence were significant irrespective of whether the adherence assay was performed in saline, or phosphate buffer [PBS] and regardless of the time of incubation.

Kearns et al.(185) detected small differences in adherence between 4 laboratory strains of \textit{C.albicans} that differed in virulence for mice, and between 3 pairs of minimally subcultured isolates from cases of oral thrush and from the mouths of healthy donors. However, such differences did not consistently show that the adherence capacities of the strains virulent for mice, or obtained recently from oral thrush, were greater than commensal strains (185). McCourtie and Douglas (186) used 7 strains of \textit{C. albicans} isolated from active infections (I strains) and 2 strains obtained from asymptomatic carries (C strains) and compared their adherence to acrylic and BEC following growth in the presence of low or high concentrations of sugars. Adhesion to BEC of all strains was reasonably similar after growth in medium containing a relatively low concentration (50 mM) of glucose. Growth in medium containing a high concentration (500 mM) of sucrose or galactose enhanced the adherence of I strains up to 5- and 11- fold, respectively. Sucrose or galactose grown C strains showed only small increases in adherence (186). The interpretation given was that I strains are able to modify their surface composition in response to growth in the presence of high sugar concentration. Such modification can enhance both their ability to adhere to surfaces and their virulence to mice. C strains lack this capability, or possess it to a lower degree, and may therefore have a lower adherability and virulence (186). Segal et al.(187) indicated that 41 isolates of \textit{C.albicans} from patients with vaginitis were significantly more adherent than 36 isolates from asymptomatic carriers. A correlation between adhesion, phospholipase production, and virulence for different isolates of \textit{C.albicans} has also been reported (188). Others (189)
showed a variation in proteinase production and adherence both among isolates of the same strain type and different strain types. *C. albicans* isolates which adhered most strongly to BEC had the highest relative proteinase activities and showed higher tissue colonisation (189). Borg and Rüchel (190) traced an acid proteinase from *Candida* spp. and they found a correlation between the ability to produce proteinase, adherence and tissue invasion. Confirmatory results were reported by Ray and Payne (191).

Soll and co-workers (192,193) reported that isolates of *C. albicans* were capable of switching heritably, reversibly and at a high frequency ($10^{-2}$ to $10^{-3}$) between two general phenotypes ('white' or 'opaque') readily distinguishable by the size, shape, and colour of colonies formed on agar at 25°C. Kennedy et al. (194) showed that white cells were found to be significantly more adhesive to BECs than opaque cells. Correlation was also found between phenotype adhesiveness and the percentage of BEC to which *C. albicans* had attached. The percentage of BECs with one or more attached *C. albicans* was approximately 90% and 50% for the white and opaque phenotypes, respectively (194). Factors which may be involved in the apparent superior adhesion of white cells to BECs is the differences in size and shape of the phenotypes [white cells are relatively round, whereas opaque cells are elongate, or bean shaped] (194).

**b. Culture Age and Viability**

The attachment of microorganisms is known to be influenced by the culture age. King et al. (180) demonstrated that the growth phase of *C. albicans* had a marked influence on its adherence ability. Stationary-phase yeasts (grown for periods longer than 18 h) attached to VEC in greater numbers than exponential-phase yeast. However, prolonged growth of the culture (for more than 24 h) did not significantly enhance adherence (180). In contrast, Segal et al. (195) showed greater VEC adherence of exponential-phase than of stationary-phase *Candida*. Others (196) showed no statistical differences in the adherence ability of *C. albicans* at different growth phases (P>0.05). Although stationary-phase yeast (grown for 24 h) showed greater adherence to BEC than
exponential-phase yeast (grown for 12h) (196). Methodologic, growth medium, cell surface hydrophobicity (197), phenotypic and strain-related differences between the above studies may account for the differences in the observed results.

Kimura and Pearsall (198) were the first to study the effect of viability of *C. albicans* on the adherence to BEC. They reported that viable yeast cells adhere better than non-viable cells, and it was found that this is related to germination rather than to cell viability. Maisch and Calderone (183) reported that heat- and formaldehyde-killed cells did not adhere as well as viable cells. Samaranayake and MacFarlane (199) found that the enhancement of adhesion of *C. albicans* to HeLa cells and acrylic surfaces disappeared once yeast cells were heat-killed prior to incubation in sucrose-containing medium. However, heat- or formalin-killed yeasts have also been reported to show diminished adherence under conditions where germination does not occur (199-201), and it is likely that such pre-treatments destroy a surface component necessary for optimal adhesion. Lee and King (202) and Klotz et al. (182) subsequently stressed that the method of fungal killing is related to adherence capacity. Gentle heat killing of the yeast results in some loss of polysaccharides and, presumably adhesins, and thus decreased adherence. Severe killing (heating for over 30 min.) will result in considerable damage to the outer surfaces of yeast cells, with concomitant loss of adhesins and consequently poor adhesion.

c. Culture Medium and Temperature of Growth

Medium composition and temperature of growth are known to affect cell morphology of dimorphic fungi including *C. albicans* (17), and, therefore, affect yeast cell-surface composition. Several workers on Candida adherence investigated the role of growth medium supplemented with different carbohydrates on the adherence process and consequently on pathogenicity of this yeast. Kearns et al. (185) reported that adherence of *C. albicans* to human BEC varied with the composition of the culture medium used to grow the fungus. Blastospores grown on synthetic agar medium adhered in significantly greater numbers than those grown on malt agar (185). Samaranayake et al. (201) showed that yeasts
incubated in media containing sucrose and glucose as the carbon source had a better adhesion to acrylic strips than control yeasts grown in sugar-free media. Growth in the presence of lactose and xylitol showed no significant difference when compared with control yeasts (201). Pre-incubation of C.albicans in the presence of a range of sucrose concentrations (from 50 to 500 mM) gave a significant positive correlation between the number of adherent yeasts to acrylic surfaces or ECs and the sucrose concentration (199,203). Douglas and co-workers (204,205) showed similar results. Galactose was the most effective carbon source and fructose the least (205). Yeast grown in the presence of 500 mM galactose showed more than nine-fold greater adherence than did control yeasts grown in medium with a relatively low concentration of glucose (50 mM) (205).

The effect of cultivation in 13 media (10 complex, and three synthetic), as well as altering growth condition, on C.albicans adhesion to BEC was studied by Kennedy and Sandin (209). Optimal adhesive activity was observed when the cells were grown in defined media (depending on the carbohydrate used). Also, significant differences in adhesion to BEC were noted when C.albicans was grown in the same complex medium from different manufacturers and in different batches of medium from the same manufacturer. Kennedy and Sandin (209) proposed the medium of Lee et al. (210) or yeast nitrogen base (Difco Laboratories) supplemented with 500 mM galactose to be used, because both media are chemically defined and produce cells of C.albicans that are highly adhesive (209).

The mechanism by which carbohydrates enhance adherence appears to be by the production of an additional fibrillar-floccular layer on the yeast cell surface (176-179). Evidence for the development of a fibrillar layer by C.albicans, under the influence of environment, came from cytochemical and ultrastructural studies carried out by Tronchin et al. (132,206). Their results indicated that adhering yeasts develop a fibrogranular surface layer visualised by a polysaccharide detection technique. They reported that adherence of C.albicans to host cells is associated with its ability to actively
rearrange substructural aspects of their cell wall coat in response to external environment (132,206). Chemical characterisation of this layer contained carbohydrates, mannose with some glucose (65% to 82%), proteins (7%), phosphorus (0.5%) and glucosamine (1.5%) (207,208).

The growth temperature of *C. albicans* has been shown to play a central role in the adhesion of this yeast to various surfaces. Lee and King (202) showed that blastospores harvested from cultures grown at 25°C adhered to VEC in significantly greater numbers than did blastospores isolated from cultures grown at 37°C. Segal et al. (195) also indicated that yeasts grown at 28°C adhered rather better to exfoliated VEC than those grown at the more usual temperature of 37°C. Kennedy and Sandin (209) found that *C. albicans* was significantly more adhesive to BECs when *Candida* cells were grown in yeast extract broth at 25°C compared to cells grown in the same medium at 37°C.

### d. Germ-tube Formation

The relation between germination and adherence has been considered by a number of investigators and the difference in adhesionability to ECs between yeasts and hyphal forms of *C. albicans* has been claimed by several authors. Kimura and Pearsall (198) demonstrated that the presence of saliva in assay mixtures incubated at 37°C resulted in increased adherence to BECs. Enhanced adherence appeared to be associated with germ-tube formation under these conditions. Adhesion was also assayed in tissue culture medium 199 which effectively induces germ-tube formation in *C. albicans* at 37°C but not at 25°C. 40–50% of the yeasts formed germ-tubes at 37°C, and adhesion was significantly greater (198). In a subsequent study, they showed a correlation between germination and increased adherence of *C. albicans* to BEC, indicating that germination or other changes in the fungi accompanying germination were responsible for enhanced adherence (211). Partial inhibition of germination by cysteine resulted in a comparably lower adherence. Competition assays with different proportions of germinated and non-germinated yeasts indicated a selective attachment of the germinated form to BECs (211).
Evidence supporting the preferential adherence of germinated yeasts came also from work by Sandin et al. (212) who demonstrated that germinated yeasts adhered to BEC more effectively than non-germinated cells, with germ-tubes being 50 times more adherent than yeast cells. In addition, germinated yeasts were more susceptible to adherence inhibition by concanavalin A than were non-germinated yeasts (212). Sobel et al. (213) found that in prolonged adherence tests, a variant strain of *C. albicans* adhered less well to VEC than its hypha-forming wild-type. Sobel and Obedeanu (214) showed that exposure of the yeast to sub-inhibitory concentrations of ketoconazole simultaneously decreased germ-tube formation and adhesion to VEC. Anderson and Odds (215) confirmed the reported reduction brought about by ketoconazole in the attachment of *C. albicans* to VEC. They showed that inclusion of high ketoconazole concentrations in the adherence assay led to a reduction in number of *C. albicans* germ-tube adhering per EC. They found that hyphae attached significantly better than germ-tube with yeast forms adhering the least (215).

e. Culture Concentration

The dependence of microbial adhesion upon culture concentration is well known. Yeast concentrations ranging from $10^2$ to $10^9$ cells /ml were tested for their influence on adherence. No detectable yeast attachment was observed at concentration below $10^4$ yeasts/ml (195, 198, 199). Kimura and Pearsal (198) reported that attachment of *C. albicans* to BEC gradually increased as the ratio of yeasts to ECs in incubation mixtures, was raised from 10:1 to 10,000 : 1. King et al. (180) showed similar results and reported that saturation of blastospore - receptor sites on the surface of VEC was not achieved at the yeast concentrations that were tested (between $5 \times 10^5$ and $5 \times 10^8$ yeast added to assay medium). They showed that yeast coadherence, i.e. adherence to other yeasts already attached to an ECs, may have been partly responsible for the high concentrations of yeasts added to the assay medium (180). In another study, Segal et al. (195) reported that varying the concentrations of both yeasts ($10^2$ to $10^9$ cells /ml) and VEC ($10^2$ to $10^7$ cells /ml) in the assay mixture revealed that adherence of yeast to ECs was saturable. They also showed that adhesion

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reached a maximum value when yeasts and VECs were incubated for two hours at a ratio 100:1, and concluded that the epithelial receptors for C. albicans were fully saturated under these conditions (195). Samaranayake and MacFarlane (199) found that above $10^4$ yeasts/ml concentration, adherence showed a significant positive correlation between the number of attached yeasts per unit area of HeLa cells monolayer and the yeast concentration in the incubating suspension. Klotz and Penn (216) showed that upon the addition of high yeast inocula, aggregation of yeasts on monolayers of human intestinal epithelium occurred rather than a random distribution of yeast cells. The greater the yeast inocula size, the greater were the aggregate sizes. This seems to support the evidence that the adherence of yeast to ECs is saturable (216).

f. Cell Surface Hydrophobicity

Cell surface hydrophobicity seems to play an important role in adherence of Candida spp. to inert materials as well as to living cells. Macura (217) divided C. albicans strains into two groups according to their hydrophobicity. The most hydrophobic strains revealed the highest adherence capacity while those less hydrophobic were less capable of adhering. Minagi et al. (218) correlated adherence of Candida spp. to dental resin materials with varying hydrophobicity. C. tropicalis bound preferentially to surfaces of increasing hydrophobicity, while C. albicans favoured less hydrophobic resin. Klotz et al. (219) showed that the adherence of Candida spp. to plastic surfaces is predominantly controlled by hydrophobic and electrostatic forces. They suggested that the electrical forces are minor to the hydrophobic ones, since adherence to a considerable extent occurs even in the presence of this repulsive force. Hazen et al. (197) showed that C. albicans cells grown at room temperature were more hydrophobic than cells grown at 37°C. Also, cell surface hydrophobicity of exponential-phase yeast cells was significantly lower than that of cells at the stationary-phase (197). These results may partly explain earlier findings that adherence of C. albicans to VEC is greater for cells grown at room temperature than cells grown at 37°C, and greater for stationary-phase cells than for exponential-phase cells (180, 202). Klotz and Penn
(216) showed that the adherence of hydrophobic yeasts was twice that of identical yeasts grown in glucose as a carbon source (yeasts grown in hexadecane as the sole carbon source tend to be more hydrophobic than those grown on glucose). Kennedy et al. (194) showed that opaque cells were twice as hydrophobic as white cells. The percentage of opaque cells bound to BEC by co-adhesion was also double that of white cells. They concluded that several factors were involved in the adhesion of *C. albicans* to plastic, and suggested that cell surface hydrophobicity was of minor importance in direct adhesion to ECs but may contribute to indirect attachment to ECs by promoting yeast co-adhesion (194,209). Hazen (220) suggested that hydrophobic interactions are not the only mechanism mediating adherence that must be considered, although they can represent the predominant force during initial contact between two surfaces (220). The predominant mechanism for adherence appears to involve adhesin-receptor interactions in which the *C. albicans* adhesin is a mannoprotein (207,208). Hydrophobic interactions are believed to contribute to adherence by maintaining the fidelity of the adhesin-receptor bonds.

2. EPITHELIAL CELL FACTORS

a. **Cell Type**

Epithelial cells represent the second partner involved in the adherence process, yeast being the first. Most studies on the adhesion of *Candida* spp. made use of a large variety of cell types including buccal (151,189,194,198,211,212), vaginal (187, 195, 200,202), uro-epithelial cells (221,222), epidermal corneocytes (181,223) and gastrointestinal epithelium (224-226). In addition to these cell types, culture cell lines include HeLa cells, kidney and cervical ECs, endothelial cells and intestine cell lines were also exploited in adhesion studies (199, 216, 227,228). Adhesion to a variety of surfaces including fibrin-platelet matrices, and inert materials such as Teflon® or denture acrylic was also studied (183,184,201).
There have been few comparisons made of relative adherence of two different cell types, particularly VECs and BECs. King et al. (180) reported that C. albicans, C. tropicalis and C. stellatoidea adhered in greater numbers to VECs than to BECs. Sobel et al. (200) found that the adherence of C. albicans to BECs was slightly greater than to VEC. Work carried out by Tomsikova et al. and Macura et al. (229,230) supports the finding of King et al. (180) that adherence was found to be more pronounced with VECs than with cells of the buccal mucosa. Ryley (231) suggested that these variations could be a reflection of the ability of the yeast to form germ-tubes under the conditions of the assay employed by different workers rather than the yeast's capacity to adhere.

Adherence of Candida spp. to human epidermal corneocytes and BEC was investigated. Collin-Lech et al. (223) reported that adherence of yeast was somewhat greater to BEC than to corneocytes (181,223). Adherence to the cells of different individuals was variable, but the ratio of values for the two kinds of cells from a single individual were quite constant (223). Ray et al. (181) confirmed that adherence of various yeast strains to BEC was higher than to epidermal corneocytes.

b. Variability

Comparative studies related to variation in adherence of Candida to BECs derived from different age groups have been performed. Cox (232,233) showed that BEC from healthy adults have no significant daily or individual change in yeast adherence. He also reported that BEC from children with oral candidosis had a greater affinity for the yeast than those from control subjects. In contrast, Tobgi et al. (234) found that BEC from children exhibited a 3-fold higher affinity for yeast, as compared with cells from adults. Davidson et al. (235) found no significant increase in the number of yeasts attached to BEC related to different age groups. However, investigating the same group of infants twice at a mean age of 2.3 and 6.2 days gave significant increase in the adherence of yeast with age (235).
Variations observed in the adherence of *C. albicans* to VEC are not unexpected in view of the influence known to be exerted by reproductive hormones on the vaginal mucosa. Segal et al. (187) reported *C. albicans* adhered in higher number to VEC collected during weeks 1 and 4 of the menstrual cycle. They related increased adhesiveness with the increased number of intermediate ECs which are rich in glycogens compared with superficial or parabasal cells. Persi et al. (236) showed that adhesion of *C. albicans* to VEC was affected by the side of the vaginal cell exposed. They reported that most of the yeast cells attached to the microridge side of the VEC, suggesting the presence of receptors that are recognised by *C. albicans* adhesin (236). King et al. (180) noticed considerable day-to-day fluctuations (in addition to individual variation) in adherence values when VEC from a single individual were tested on a daily basis.

It is clear that variability of both host and microbial cells influence the results of adherence studies. Such variability may reflect differences in the number or nature of available receptors on the EC membrane (176-179).

3. ENVIRONMENTAL FACTORS

a. Hydrogen-ion Concentration and Carbon Dioxide

Optimal pH for adherence of both bacteria and yeast is reported to be in the range of 6 to 8 (175,176-179). Some authors (180,200) reported that adhesion of *C. albicans* to VEC was optimal at pH 6 to 7, while others (203) showed that adhesion of *C. albicans* was slightly enhanced at pH 7 and highly enhanced at pH 3, with HeLa cells as the substrate. Persi et al. (236) showed that adhesion of 4 strains of *C. albicans* to VEC appeared to be affected by the pH and CO₂ levels present in the adhesion assay, with greater adhesion ability when the yeast cells were incubated with VEC at pH 5 in PBS in ambient air supplemented with 10% CO₂.

Mehentee and Hay (237) showed that adherence of *C. albicans* strains to murine gastrointestinal mucosal surfaces was affected by the pH of the medium. Binding between *C. albicans* strains and
stomach mucosal cells fluctuated as the pH of the medium was raised from pH 1.2 to pH 3.4. Optimal adherence of two strains used by these workers to jejunal mucosal surfaces occurred at neutral pH (237). Adherence was also found to be dependent on the strain used, and on the cellular arrangement, and the site of origin of the mucosal surface (237).

b. Antibodies

The influence of antibodies to Candida on mucosal adherence has been examined. Kimura and Pearsall (198) suggested that antibodies to Candida inhibit in vitro adherence to BEC. Epstein et al. (238) showed an inverse correlation between the titer of salivary IgA [a relatively minor immunoglobulin in serum] and the adherence of Candida in the presence of IgA-bearing saliva. However, adherence increased in only 4 of 13 experiments following partial removal of salivary antibodies to Candida by immunoprecipitation of IgA and in only 4 of 20 experiments following immunoprecipitation of all antibody classes (238). Vudhichamnong et al. (239) reported that adhesion of C. albicans to BEC was inhibited by human breast milk IgA. The inhibitory effect was maximal after pre-incubation of yeast cells and IgA for 1.5 hours and was concentration dependent on IgA (239). This finding may be relevant to the lower incidence of thrush in breast-fed than in bottle-fed infants (17).

Vuddhakul et al. (240) studied the effect of human neutrophils on the adherence of C. albicans to dacron fibre microcolumns, and showed that adherence of C. albicans was significantly suppressed after interaction with human neutrophils. The adherence-inhibiting properties of neutrophils were shown to reside in their cytoplasmic granules and granular enzymes. Furthermore, oxygen radicals produced by the hypoxanthine - xanthine oxidase reaction were capable of suppressing the adhesion of Candida (240). Dose-response with H$_2$O$_2$ and $\beta$-glucoronidase [can cause degradation of oligosaccharides in the fungal cell wall (241)] showed that lower concentrations of these agents inhibited adherence without affecting viability of C. albicans (240).
The role of saliva on the adherence of *C. albicans* to human ECs and acrylic strips has been studied. McCourtie and Douglas (205) reported that adherence of *C. albicans* to acrylic strips was inhibited when saliva-treated strips were used in assays or when the yeasts were suspended in saliva. Samaranayake and MacFarlane (203) showed that adhesion of *C. albicans* to HeLa cells increased significantly under the influence of salivary pellicle. Kimura and Pearsall (198) showed that viable *C. albicans* cells, pre-incubated in saliva for 90 min at 37°C before being washed and mixed with ECs in PBS, adhered better than non-viable yeasts or yeasts pre-incubated in PBS. Further examination showed that the enhanced adherence of *C. albicans* observed after incubation with saliva is more a reflection of the ability of the yeasts to form germ-tubes, rather than a requirement for prolonged interaction between fungi and ECs (198).

d. Relation of Normal Body Flora to Candida Adherence

Evidence suggesting involvement of the indigenous bacterial flora in the colonisation process of *C. albicans* came first from work carried out by Liljemark and Gibbons (242). They showed that cells of *C. albicans* adhere in lower numbers to ECs from the tongue or cheek of conventional rats rather than to those from germ-free rats. In addition, mouth and gut colonisation by this yeast in gnotobiotic mice was shown to be suppressed by mixed human salivary bacteria and strains of *Streptococcus salivarius* and *S. mitior* (242). Sobel et al. (200) reported a reduction of *C. albicans* adhesion to VEC by pre-incubating the VEC with two vaginal isolates of *Lactobacillus* before incubating with the yeast. Samaranayake and MacFarlane (203) showed that *S. salivarius* and *S. mitior*, but not *S. mutans* reduced the adherence of *C. albicans* to HeLa cells in vitro. Makrides and MacFarlane (243), showed that cell suspensions and cell suspension supernatants of *Escherichia coli* and *Klebsilla aerogenes* significantly enhanced candidal adherence to HeLa cells. In contrast, *Staphyloccus aureus*, *S. mitior* and a strain of *S. sanguis* significantly reduced adherence. Cell suspensions and cell suspension supernatants of
S. milleri and a second strain of S. sanguis had no significant effect on candidal adherence to HeLa cells (243). In a subsequent study, Makrides and MacFarlane (244) showed that pre-incubation of HeLa cell monolayers with E. coli cell suspensions led to a significant increase in the adherence of C. albicans to these ECs. Centeno et al. (245), however, showed that pre-incubation with piliated, but not with non-piliated, strains of E. coli and K. pneumonia enhanced the subsequent attachment of C. albicans to the ECs. Acrylic denture strips pre-coated with S. salivarius cells had a significantly lower number of Candida cells attached to them than the non-coated strips (201).

The mechanisms by which indigenous microflora interfere with the adherence of C. albicans to ECs have been investigated. Kennedy and co-workers (246-248) suggested that the indigenous microflora reduces the mucosal association of C. albicans by forming a dense layer of bacteria in the mucous gel bathing these epithelial layers, surpassing yeast cells for adhesion sites (epithelial receptors). Another possibility is the production of inhibitory substances, such as volatile fatty acids, which modify Candida adhesion(s) or mucosal receptor(s), or both, thus making the occurrence of the yeast-mucosal association difficult (246-248). Differences observed in the capabilities of various members of the indigenous microflora which affect adherence of Candida may be explained on the basis of differences in their abilities to adhere to mucosal surfaces. Thus, Streptococcus salivarius and S. mitior, which have been shown to adhere well to oral mucosal surfaces, were able to suppress C. albicans in the oral cavity, whereas S. mutans, which is known to attach poorly to oral ECs, had little effect on yeast colonisation (242). The mechanisms proposed for the in vitro studies can also be applied for the in vivo one. Thus, C. albicans can readily associate with and pass through the gut wall of infant mice, which lack a complete bacterial flora, including the dense microbial population in the mucous gel (249), initiating a systemic infection (250). In contrast, adult mice, that have fully developed indigenous flora, are resistant even to colonisation (250). Protection against Candida-mucosal association and subsequent dissemination has been reported in
adult hamsters having an indigenous wall-associated microflora (248). It is suggested that mucosal association of C. albicans is reduced by competition for adhesion sites and physically preventing the larger yeast cells from penetrating mucous gel. Evidence to support this hypothesis was also gained in the same study by scanning electron microscopy and disassociation studies (200, 251). It seems likely that there are several inhibitory mechanisms produced by indigenous microflora which may be acting together in suppressing the colonisation and dissemination of Candida from the gut.

Other environmental factors which affect the adhesion of C. albicans to various surfaces include time of contact (180, 195, 199), presence of secretions such as hormones and mucous (187, 222, 224-226), and age-related alterations (232-235).

4. CHARACTER OF INANIMATE SURFACES

Catheters and denture base resin materials are important inanimate substances closely associated with candidosis (17, 178). Adherence of Candida spp. to plastic intravenous catheters has been reported by Rotrosen et al. (184). Few studies exist dealing with the properties of inanimate substrates in relation to Candida adhesion. Most investigations look at the adhesion process as affected by parameters related to the yeast with only slight reference being made to parameters related to inanimate substrates (178).

Minagi et al. (218) studied the relationship between the surface free energy of denture base resin plates and adherence of C. albicans (a relatively hydrophilic spp.) and C. tropicalis (a hydrophobic spp.). Close relationships were observed between the surface free energies of resin plates and adherence of these two spp. Increasing surface free energy of resin plates resulted in an increase in the adherence of C. albicans but decreased the adherence of C. tropicalis. They also investigated the change of the free energy accompanying the adherence per unit area of adherence (\( \Delta G_a \)) and found that a decrease of \( \Delta G_a \) resulted in an increase adherence of both C. albicans and C. tropicalis (218).
Another two properties of inanimate surfaces relevant to adhesion are hydrophobic interactions and electrostatic forces. Minagi et al. examined the effects of hydrophobicities of substrate surfaces using two species of *Candida* (218). They showed that hydrophobic interaction takes part in the adherence of both spp. to surfaces. Klotz et al. (219) showed that hydrophobic forces predominantly control the adherence of *Candida* spp. to plastic surfaces. Furthermore, electrostatic interactions occur simultaneously, creating a condition of negative cooperative binding. The electrical forces are minor to hydrophobic forces, since considerable adherence occurs even in the presence of this repulsive force (219).

Wettability of the inanimate surfaces is another parameter associated with attachment of both microbial and mammalian cells to various surfaces (178). However, studies on the effect of this property on *Candida* adhesion are lacking.

Other factors affecting the adherence of yeast to inanimate surfaces include surface area of the material as well as the form in which it is manufactured. The adherence of *C. albicans* to braided and monofilament sutures was compared by scanning electron microscopy. *C. albicans* adhered to braided more than monofilament sutures. Spaces between the filaments provided a trapping site for the yeast and other suspended materials and braided sutures provided higher surface areas. Furthermore, the place at which the sutures were cut provide an attachment area for the yeast (178).

Obviously, the mechanism/s by which microorganisms adhere to inanimate surfaces involve many overlapping phenomena, and that more studies on the adherence of *Candida* spp. to such surfaces should be undertaken.
CHAPTER FOUR

SPECIFIC CHEMICALS AND MECHANISMS INVOLVED IN CANDIDA ADHERENCE
Specific Chemicals and Mechanism/s Involved In Candida Adherence

The initial contact between microorganisms and epithelial tissues is probably a random and instantaneous process which depends on general rather than specific surface characteristics (175-179). This type of adhesion is reversible serving to stabilize the microbial cell at site and prepare for the more permanent association between the microorganism and the ECs (178). This permanent interaction, known as irreversible adhesion, is a specific interaction mediated by (macro)molecules on the microbial surface (referred to as adhesins) combined with complementary structures on the host cell surface (referred to as receptors) (178).

I. Adhesins

Ultrastructural evidence indicates that specific interaction between Candida and ECs is mediated by a floccular-fibrillar adhesin layer present on the outer surface of the yeast (132,153,176-179, 186,205-209). Montes and Wilborn (252) were the first to observe the presence of this floccular material on the outer surface of C.albicans cell wall. Description of this layer in literature varied. Some workers described it as thin filamentous structure (2-10 nm in diameter) (253-255). Others reported it as a thick (approximately 100-400 nm) fuzzy, amorphous cell wall coat (206,209). This adhesin layer is reported in some studies as being unevenly distributed on the cell surface or localised only at an adhesive site (115,205,209,256). Other studies suggest that it has an ordered alignment around the cell wall (206). This may be due to differences in media and carbon source and/or concentrations used by different workers.

Experimental evidence exists to indicate that the degree of development of this outermost layer is related to the adherability (186,204,205,255,256). Cells with thicker layers are more adherent. Tokunaga et al.(257) showed that the adhesion of
Candida cells to BEC corresponded with increased density of the fibrillar structure of the outermost Candida cell wall layer. Another study associated adherence with the condensation or disposal of this layer, facilitating contact between ECs and the deeper layer of the fungal wall (256). In several SEM studies it was demonstrated that fibrils mediate Candida attachment to ECs in vitro (258-260). Clearly, this fibrillar-floccular layer mediates Candida adhesion.

The chemical nature of Candida adhesin/s is unknown. Some reports propose that C. albicans produce more than one adhesin (209, 261,262). Based on different experimental approaches, a number of compounds have been suggested as possible Candida adhesins (Table 4).

a. Sugars and glycoproteins

Sugars and glycoproteins, particularly mannoproteins, received major attention by workers as possible Candida adhesins. This is understandable as they form the outermost layer of the yeast cell wall (176-179) [See Chapter Two]. The results of many studies revealed that the inclusion of particular sugars and sugar derivatives in the assay medium resulted in the blockage of adherence of Candida cells to ECs and to synthetic surfaces.

McCourtie and Douglas (205) showed that the adherence of yeast to acrylic surfaces in vitro was increased after growth of the yeast in a medium containing high levels of different sugars, particularly galactose. Douglas et al. (204) confirmed this result on C. albicans adherence to BECs. Samaranayake and MacFarlane (265) showed that various strains of C. albicans pre- incubated in a medium containing glucose, sucrose, galactose, xylitol or maltose exhibited enhanced adherence to HeLa epithelial monolayers and BECs. The most effective sugar in this respect was maltose and the least effective glucose, although different patterns were obtained with other strains. The same authors concluded that the medium sugars modify the adhesive properties of Candida cells. In these studies it was noted that adhesion was directly proportional to the sugar concentration. McCourtie and
<table>
<thead>
<tr>
<th>Inhibitors used</th>
<th>Proposed adhesin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosamine, chitin-soluble extract, N-acetylglucosamine, mannosamine</td>
<td>Chitin</td>
<td>195</td>
</tr>
<tr>
<td>Mannose, α-mannosidase, tunicamycin, α-methyl mannosidase, concanavalin A,</td>
<td>Mannan/</td>
<td>179, 215,</td>
</tr>
<tr>
<td>D-methylmannopyranoside</td>
<td>mannoprotein</td>
<td>255</td>
</tr>
<tr>
<td>Glucose, glucan</td>
<td>Glucan</td>
<td>255, 263</td>
</tr>
<tr>
<td>Papain, pronase, pepsin, trypsin, chymotrypsin</td>
<td>Protein</td>
<td>255, 263</td>
</tr>
<tr>
<td>Sterols</td>
<td>Lipid</td>
<td>264</td>
</tr>
</tbody>
</table>
Douglas (266) found that *C. albicans* grown on 500 mM galactose adhered to acrylic surfaces at a maximal linear rate throughout an incubation period of 1 h, whereas nonlinear adhesion rates were observed with cells grown on 500 mM sucrose, 50 mM glucose or galactose.

Thus, Segal et al. (195) showed that GLcNAc, glucosamine and mannosamine, but not mannan, glucan, glucose, α-methylglucose, mannose or α-methylmannose could block the adhesion of *C. albicans* to VEC. The results on the effect of mannose are contradictory. Centeno et al. (221) showed that mannose, but not glucose, significantly decreased the attachment of *C. albicans* to ECs. Other investigators could not trace any blocking effect for mannose added into the assay medium (200, 202, 263, 267). A number of disaccharides viz. maltose, sucrose, lactose, cellobiose and trehalose were effective but the monosaccharide galactose was not (151). Most effective were amino-sugars like mannosamine, glucosamine and galactosamine (223).

The lectin [these are proteins or glycoproteins with multiple sugar-binding sites] Con A, which binds to α-linked D-mannose residues, was used by a number of workers to investigate the role of mannoproteins in adherence. Most of these studies indicated that this wall component acts as a *Candida* adhesin (179, 268). Tkaez et al. (269) used fluorescein-conjugated Con A to locate mannan-rich areas of the cell surface. Whole cells of *C. albicans* were found to stain strongly with fluorescein-conjugated Con A but became resistant to this stain in the presence of lectins such as soybean agglutinin. Maisch and Calderone (183, 270) showed that cell surface mannan may play an essential role in the adherence of *C. albicans* to the fibrin-platelet matrices which form on the endocardium of heart valves. Sandin and associates (212, 271) revealed that Con A inhibited adherence when it was used for the pre-treatment of either the yeast or the ECs. Adherence was restored by pre-incubating Con A with a mannose derivative, but not with other sugars. Lectins that do not recognise mannose apparently did not affect adherence. Confirming results were reported by Rossano and Tufano (272).
The treatment of *C. albicans* with tunicamycin, an antibiotic inhibiting protein glycosylation and, at low concentrations, inhibits mannoprotein synthesis but not chitin or glucan synthesis, interfered with the adherence of this yeast (273). Douglas and McCourtie reported that the addition of tunicamycin, at the early stationary phase, inhibited the formation of the fibrillar layer with a consequential decrease in adherence to BEC [over 60% reduction as compared with control yeast cells] (274).

The role of the protein portion of mannoprotein in adherence has received little attention. There is evidence to suggest that this portion may serve as an adhesin. Some reports suggest that the protein portion of the mannoprotein complex is more important than the carbohydrate moiety in mediating adherence (177). Evidence to support the role of proteins as *Candida* adhesins comes from experiments showing that *C. albicans* cell adherence decreases following exposure to heat (resulting in protein denaturation) or various proteolytic enzymes (200,202). Maisch and Calderone (270) showed that pre-treatment of *C. albicans* with pronase, trypsin and chymotrypsin led to a reduction of yeast cell adhesion to fibrin-platelet clots. Further supporting evidence came from an investigation which showed that pre-treatment of an extracellular polymer [EP] [identified subsequently as mannoprotein (207,208)] from *C. albicans* with heat, dithiothreitol or certain proteases, but not α-mannosidase or sodium metaperiodate, destroyed, partly or entirely, its ability to inhibit yeast adhesion to ECs (179). The binding affinity of *C. albicans* pseudohyphae to neutrophils was reported to be reduced under the influence of a protein component derived from this yeast (275). Other workers reported that binding of *C. albicans* to fibrinogen was diminished when the fungus was pre-treated with 2-mercaptoethanol alone or together with pronase, with α-mannosidase, or trypsin, but not with pronase alone nor with chitinase (276). Recently Tronchin et al. (277) gave evidence for the presence of four specific proteins involved in *C. albicans* germ-tube adherence to plastic. These results clearly demonstrate that the protein portion, of the mannoprotein layer, influences the adhesion process and may partly explain why *Candida* adhesion is not always inhibited by mannose, methyl-α-D-mannoside or
mannoprotein preparations devoid of protein portion (195,200, 202).

A role for chitin in the adhesion of C. albicans was suggested by Segal and co-workers (195,224,278). Chitin, its constituent GLcNAc and chitin soluble extract (CSE) inhibited yeast adhesion to VEC. Lehrer et al. (278) observed that both GLcNAc and CSE also reduced the infection rate in a rat model of vaginitis. Attempts to characterise the CSE showed that it consists of two fractions : FI and FII, of which only FI exhibited inhibitory activity. Chemical analysis of CSE and its two fractions showed that CSE contains over 70% of proteins, concentrated mostly in FII. In addition, 3% of amino-sugars were detected both in the whole CSE and in FI, and lipids in the unfractioned CSE and both fractions (278).

b. Lipids

The role of lipids in Candida adherence has so far received limited attention, despite the fact that lipids are major constituents of the cell surfaces. There are some relevant findings in the literature regarding the role of lipoteichoic acids in the adherence of bacteria to ECs (175,280). The lipid class glycosphingolipids, normally detected in small amounts in plasma membranes of animal cell, has been reported to be the site at which the fimbriae of Gram-negative bacteria adhere (178).

Experimental evidence for the involvement of lipids in adherence of Candida spp to BEC has been reported (151,264). Total lipids extracted from C. albicans and other Candida spp. significantly blocked in vitro adherence of yeast cells to BECs. The constituent lipid classes of C. albicans were then isolated in a pure form and tested individually. The results indicated that various phospholipids viz. diacylglycerophosphocholines, diacylglycerophosphoethanolamines, diacylglycerophosphoglycerols, diacylglycerophosphoinositols, and diacylglycerophosphoserines were highly active in blocking adherence (264). Similar results were obtained with sterols and steryl esters, but triacylglycerols and fatty acids did not have any significant
effects. Lipids classes found efficient in blocking adherence are those known to be constituents of the plasma membrane. In another study, C. albicans glycolipids, namely ceramide monohexosides and ceramide dihexosides from yeast form and sterylglycoside from mycelial forms, were also found to block adherence in vitro (151). Such glycolipid classes most probably also occur in the plasma membrane. The results of the same study have further shown that yeast wall lipids and lipids from ECs were also significantly effective in inhibiting adherence.

II. Cell surface receptors of Candida adhesins

Compared to information available on Candida adhesins, reports on its receptors are sparse and the receptors on various tissues to which this yeast adheres are not well characterised (177,178). Experimental evidence suggests that cell surface receptors of Candida adhesins are carbohydrates [including glycoproteins] and lipids (179,264,267).

Critchley and Douglas (208) observed that Candida adhesion is mediated by more than one receptor. They showed that glycosides containing L-fucose, GLcNAc and possibly D-mannose can all function as epithelial receptors for different strains of C. albicans (Table 5). Evidence for their suggestions was partly derived from experiments utilising various lectins. The fucose-type receptors may be most commonly required for Candida adhesion (208). Based on inhibition studies carried out by a number of workers, it is suggested that the natural epithelial receptor is not an L-fucose residue as such but probably a larger molecule with a particular stereochemical configuration (208,254,281).

Rotrosen et al. (282) suggested that the host cell receptor for Candida adhesion is fibronectin. Skerl et al. (267) investigated the binding of C. albicans to human fibronectin in vitro. Their results indicated that around 40% of the yeast cells adhered to fibronectin, with greater adherence achieved at 30°C than at 4°C and with viable cells than heat-killed cells. It was also demonstrated that the adherence process was blocked when yeast cells were pre-treated with enzymes chymotrypsin, pronase
or papain but not with pepsin (267). Experimental evidence for a possible role of fibronectin as a receptor for the binding of Candida to ECs was provided (267). Thus, they demonstrated the presence of fibronectin in BECs and VECs. Furthermore, they reported that fibronectin pre-treated yeast cells showed lesser adherence to ECs than non-treated ones (267).

The clearest demonstration of the role of lipid components as receptors for microbial adhesins came from the work of Sato and co-workers (283). These researchers reported that sterol-containing liposomes inhibited haemagglutination by Bordetella pertussis. Further evidence for the interaction of bacterial adhesins with lipids is provided from investigations by Gibbons and colleagues (284,285). Other workers (151,264) showed that total lipids extracted from BEC blocked the adherence of C.albicans by 53%. Four lipid fractions; sterols, steryl esters and two phospholipids [diacylglycerophosphocholines and diacylglycerophosphoethanolamines] were shown to be highly effective in blocking adherence of C.albicans in vitro. Table 5 list possible surface receptors of Candida adhesins.

III. Mechanism/s of Candida Adherence

Candida may adhere to epithelial surfaces through either direct interaction, i.e. yeast cells to epithelial tissues, or indirectly through bridging involving intermediate cells/components, or both.

a. Direct interactions

Direct Candida - epithelial cell adhesion seems to pass through two phases prior to cell invasion: non-specific interactions [referred to as loose or reversible] (286) and specific interactions [referred to as irreversible, tight or intimate] (155, 256,286) brought about by the molecular interaction of the adhesins and receptors. The reversible phase lasts for about 20 minutes, thereafter the cells bind irreversibly (286).
Table 5

Possible surface moieties which may serve as receptors for *Candida* adhesins

<table>
<thead>
<tr>
<th>Possible Receptor Moieties</th>
<th>Epithelial Cell type</th>
<th>Inhibitors</th>
<th>References</th>
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<tr>
<td><strong>Sugar moieties</strong></td>
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<tr>
<td>Fucose</td>
<td>Buccal</td>
<td>L-fucose,</td>
<td>208, 254, 281</td>
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<td></td>
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<td>Lectin from</td>
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<td></td>
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<td><em>Lotus tetragonolobus</em></td>
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<td>Vaginal</td>
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<td>Mannose</td>
<td>Buccal</td>
<td>Con A, D-mannose,</td>
<td>212</td>
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<tr>
<td></td>
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<td>methyl- α-D-mannoside</td>
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<td>N-acetyl-D-glucosamine</td>
<td>Buccal</td>
<td>N-acetyl-D-glucosamine,</td>
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<td>Intestinal</td>
<td>-</td>
<td>261</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Buccal</td>
<td>-</td>
<td>267</td>
</tr>
<tr>
<td><strong>Lipid moieties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipids, sterols</td>
<td>Buccal</td>
<td>Phospholipids, sterols</td>
<td>264</td>
</tr>
</tbody>
</table>
Candida and cell surfaces to which they adhere have a net negative charge and repel each other (287). Once in close proximity, other forces of attraction and repulsion exert their effects, counteracting electrostatic repulsion and probably initiating cationic bridges (288). The cationic bridges may culminate in the formation of a definite loose association, which is followed by tight or intimate adhesion. Ultrastructural studies on colonisation and invasion of oral epithelium carried out by Howlett and Squier (256) showed that the adherence of yeast to the superficial cells of the oral mucosa appeared to involve intimate contact between the ECs surface and the deeper layers of the C. albicans cell wall. This close contact is maintained during invasion where a "close seal" between the invading hyphae and the surrounding EC envelope was observed (155,256). Marrie and Costerton (155) observed a tight adhesion between yeast cells and mucosal ECs where no space could be seen between the host and yeast cell. Results reported in these studies do not demonstrate unequivocally that a distinct phase of reversible adsorption need precede adhesion of yeast to host cells. Possibly, in vivo, micro-organism may produce adhesins only after they become reversibly adsorbed to the cell surface (36,108). In this respect Tronchin et al. (206) showed that C. albicans cells undergo ultrastructural modifications in their cell wall coat during adhesion and subsequent invasion of the yeast to ECs and tissues.

The mechanism/s responsible for attachment and penetration of vascular endothelium have been studied. These investigations proposed different mechanisms. Klotz et al. (219) showed that within 15 min. C. albicans could attach tightly to the porcine vascular endothelium tissues, with a partial disruption of the endothelial surface. Severe damage to the endothelium was observed by 30 min., and by 60 min. yeast cells had penetrated deep into the porcine endothelial cells and were nearing the basement membrane (219). This penetration occurred with only viable cells which suggests that it is mediated by enzymatic activity. Although Rotrosen et al. (282) proposed a similar sequence of events for the attachment and penetration of endothelial cells by Candida to that proposed by Klotz et al.,
the mechanism of adhesion and penetration proposed was different. They suggested that phagocytosis contributes to the initial entry of Candida into the endothelial cell. An alternative mechanism for the adhesion of C. albicans to endothelial cells is attachment to damaged endothelium. Klotz and Maca (289) simulated endothelial injury by eating bovine vascular endothelial cells with EDTA, urea, or thrombin. These treatments led to a marked reversible contraction of the endothelial layer, resulting in exposure of subendothelial extracellular matrix (ECM). The authors showed that Candida cells adhered more to contracted monolayers than to confluent monolayers ($P<0.01$) but preferentially adhered to the ECM. This suggests that exposure of the ECM in vivo may contribute to the establishment of metastatic Candida lesions (289). Another way for micro-organisms to adhere to injured endothelial tissues is through binding to a fibrin-platelet matrix. Calderone and co-workers (290,291) investigated Candida endocarditis using a rabbit model and characterised the development of vegetation formation following intravenous injection of C. albicans cells. Adherence to fibrin-platelet-erythrocyte deposits took place within 30 to 90 min. (290). Phagocytes were abundant within the vegetation, and could be seen with ingested yeast cells. Many of the Candida appeared capable of surviving phagocytosis and produced germ-tubes. The infection progressed with Candida cells developing into large colonies of abundant pseudohyphae and blastospores. These fungal elements were apparently protected within the vegetation by the continued deposition of host elements (290). Further studies are required to clearly define the nature of these mechanisms and their relative importance.

b. Indirect interactions

Microorganisms may adhere to specific tissues by first binding to intermediary microbes, host cells and/or "bridging" ligands derived from host and microorganisms (178). C. albicans interacts with mucosal surfaces from oral, GI and urogenital systems by similar mechanisms including co-adhesion to adherent fungi, co-adhesion to adherent bacteria, and entrapment in the mucous gel overlying the epithelium (155,261,262,292).
Pope and Cole (255), examining the stomach mucosa of infant mice, revealed that at early times after yeast inoculation Candida cells were adhering to both keratinised squamous epithelia and columnar secreting epithelium. At later stages Candida were also seen attached to the secreting epithelial tissues surrounded by mucous with some cells nearly covered by the mucous layer. Candida cells were also observed to be associated with lactobacilli adherent to the keratinised epithelium (255). These results suggest that adhesion of C. albicans to the stomach mucosa is achieved through several mechanisms, including both direct and indirect attachment.

The association of C. albicans with small intestinal mucosa has been studied and is reported to be mediated by several different mechanisms (250,255,261,293). Cole and co-workers showed C. albicans to be in contact with intestinal microvilli 1 hour after oral-intragastric challenge of infant mice (226,250,293). Many yeasts were also seen frequently in association with the mucous and appeared to be attached to, embedded in, or covered by a layer of mucous. Yeast cells were capable of progressive extracellular digestion of the intestinal mucous barrier and microvillus layer, followed by intracellular invasion of ECs. Kennedy et al. (247,248,261) showed that C. albicans associates with the large intestinal mucosal surfaces of antibiotic-treated mice by similar mechanisms. However, control mice possessing an indigenous wall-associated bacterial flora did not behave in the same way. Examination of caecal mucosa from antibiotic-treated mice showed that C. albicans associated with the mucosal surfaces by at least five distinct mechanisms (248,261).

Similarly examination of attachment of C. albicans to vaginal mucosa revealed that yeast cells could attach to the epithelium by several adhesion and association mechanisms. These include Candida direct adhesion and co-adhesion to adherent organisms (155,292) [Table 6].
In addition to the mechanisms so far described, there are several other mechanisms whereby micro-organisms could indirectly bind to host tissues. These mechanisms utilise host-derived "bridging" ligands, such as fibronectin and plasma proteins (e.g. fibrinogen), and/or "bridging" cells (178). The role of fibronectin in the adhesion of C. albicans has already been mentioned above (294). Interaction of Candida with human plasma proteins are of particular importance in relation to adhesion, due to the fact that exposure of C. albicans to mammalian serum results in blastospore - hyphal conversion and/or changes in cell surface structure (153). Bouali et al. (295) carried out cytochemical and ultrastructural studies on the binding of human fibrinogen to C. albicans in vitro. These workers reported that purified human fibrinogen appeared to bind more strongly to hyphae than to yeast forms. This was confirmed by others (296). Page and Odds (297) evaluated the propensity of 3 plasma proteins, albumin, fibrinogen and transferrin, to bind to Candida spp. in vitro. They showed that all the 3 proteins tested bound with high avidity to germ-tubes formed by C. albicans [with fibrinogen bind more readily than the other two proteins] but did not bind to blastospores of C. albicans or other pathogenic spp., even to parent blastospores bearing germ-tubes (297). Although Tronchin et al. (298) proposed that fibrinogen bound to germ-tubes acts as a ligand in binding to fibronectin on host cell surfaces and/or as a mechanisms for providing the yeast with a protein coat that could protect it from host immune systems, Page and Odds suggested that adhesion via plasma protein intermediates is more likely to be a secondary than a primary mechanism (297). Table 6 list possible mechanism/s of Candida adherence and their nature.

Obviously, chemotherapeutic agents may affect the mechanism/s by which Candida spp. adhere to mucosal cells and inanimate surfaces in vitro and in vivo. Therapeutic agents that have been studied in relation to their effect on Candida adherence may be divided into two categories: (a) drugs used for the prevention and control of infection, and (b) therapeutic regimes generally associated with potentiation of candidosis. A number of studies have been directed towards the role of agents in the
first category. These include antifungal agents \((214, 215, 308-312)\), chlorhexidine \((300-303)\), formaldehyde -releasing agents such as noxythiolin, polynoxylin and taurolin \((304-307)\). Studies related to the effect of the second category include investigations on glucocorticoids \((196)\), antineoplastic agents and X-irradiation \((299)\).

Publications regarding the effect of various antifungal agents on adherence of *Candida* spp. to host cells are limited in number and contradictory. It is the purpose of this study to carry out a comprehensive investigation into the effect of AMB, NY, MN and 5-FC on the *in vitro* adherence of three *Candida* spp. to BECs. The effect of two new antimicrobial drugs, octenidine and pirtenidine, on the adherence of *Candida* spp. was also investigated *in vitro*.

Murine intestinal disks and an infant mouse model was also used to establish GI and systemic candidosis and explore the effect of saccharides, known to block adherence of yeast to ECs *in vitro*, on the GI transient colonisation and dissemination and as a possible means for prevention of candidal infection.
### Table 6
Mechanisms of *Candida* adherence and their nature

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Active/Passive</th>
<th>Specific/Nonspecific</th>
<th>Direct/Indirect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entrapment in tissue</td>
<td>-/+</td>
<td>-/+</td>
<td>+/-</td>
<td>255, 261, 293, 294</td>
</tr>
<tr>
<td>Nonspecific interaction</td>
<td>+/+</td>
<td>-/+</td>
<td>+/-</td>
<td>218, 219, 237, 261</td>
</tr>
<tr>
<td>Coadhesion to adherent organisms</td>
<td>+/-</td>
<td>+/+</td>
<td>-/+</td>
<td>209, 221, 261</td>
</tr>
<tr>
<td>Specific interaction (Adhesin-receptor)</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>195, 211, 212 223, 261, 278, 294</td>
</tr>
<tr>
<td>Germ-tube penetration</td>
<td>+/-</td>
<td>+/+</td>
<td>+/-</td>
<td>155, 213, 256, 282</td>
</tr>
</tbody>
</table>

**+** Functional

**-** Not functional
CHAPTER FIVE

MATERIALS AND METHODS
1. ORGANISMS:

Three species of Candida were used in this study: *C.albicans*, *C.tropicalis* and *C.kefyr*. Three strains (all are serotype A) of the first species were investigated, *C.albicans* ATCC 10231 isolated from bronchomycosis was obtained as lyophilised samples from the American Type Culture Collection, Rockville, Md. The other two strains, *C.albicans* KCCC 14172 and *C.albicans* KCCC 13878, as well as *C.tropicalis* KCCC 13605 and *C.kefyr* KCCC 13709, were isolated on Sabouraud dextrose agar [SDA] medium (Difco Laboratories, Detroit, Michigan) from the oral cavities of patients undergoing head and neck radiation therapy at the Kuwait Cancer Control Center. Samples were taken from two sites of the patients oral cavity: gingival sulci and pharyngeal portion (319). These were then inoculated into Sabouraud broth medium (Difco) containing 50 mg/L chloramphenicol (Sigma Chemical Company, Saint Louis, Missouri). Tubes were incubated at 37°C for up to 48 h, when streaking onto SDA medium (Difco) was performed to obtain isolated colonies.

Identification of the yeast species was based on germ-tube formation and the use of the API 20 C system. Germ-tube formation was performed by adding an inoculum of approximately $10^4$ cells/ml to tubes containing 5 ml of new born calf serum (Gibco, Grand Island, N.Y.) and incubated in a shaking water bath at 37°C for 2-5 h, and examined by phase contrast microscope (x 400) for the formation of germ-tubes (200). The isolate was considered to have formed a germ-tube when a narrow tube (at least 2μm long) extended from the mother cell. Stock cultures were maintained on SDA (Difco) and stored at 4°C.

2. ANTIFUNGAL DRUGS AND CHEMICALS:

Antifungal agents used were: Amphotericin B (AMB), Nystatin (NY), Miconazole nitrate (MN) and 5-Fluorocytosine (5-FC) [Figure 10]. All drugs were purchased from Sigma chemical company.
Octenidine hydrochloride \([N, N^\text{-}(1.10\text{-decanediyldi-1[4H]-pyridinyl-4-yldene})\text{ bis-(1-octanamine) dihydrochloride}]\) and pirtenidine \([N-(1\text{-octyl-4[H]}\text{-pyridinylidene})\text{ octanamine monohydrochloride}]\) are new drugs developed by Sterling-Winthrop Research Institute as antimicrobial or antiplaque agents (Figure 10). These drugs were kind gifts from Sterling-Winthrop.

The following substances were tested for their possible interference with \textit{C. albicans} adherence to intestinal disks and the transient GI colonisation and dissemination of this yeast in infant mice: mannose, N-acetylglucosamine (GLcNAc) (Sigma Chemical Company) and chitin soluble extract (CSE). The latter was prepared by suspending purified chitin powder from crab shells (Sigma product number C 3641) (2\% w/v) in phosphate buffered saline (PBS) with shaking at room temperature for 5 h (187, 195, 278). The supernatant designated as chitin soluble extract (CSE) was then removed, dialysed overnight at 4\(^{\circ}\)C against sterile water and lyophilised. The yield of CSE in most of the preparation was 0.04 - 0.1\% of lyophilised material /ml of supernatant.

3. MEDIA AND BUFFER SOLUTIONS:

The following media (g/L) were used and sterilised at 121\(^{\circ}\)C for 15 min (unless otherwise specified). When required, solidified media were obtained by adding Bacto-Agar (Difco) to give a final concentration of 2\% (w/v). All media were prepared from Difco products.

**Minimal Medium (Without carbon source)**

- Ammonium sulphate 1.0
- Monopotassium sulphate 1.0

pH adjusted to 6.0 ± 0.1
Figure 10. The structure of the antifungal drugs used throughout the work.
**Sabouraud Dextrose Broth [SDB]**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neopeptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20.0</td>
</tr>
</tbody>
</table>

pH adjusted to 5.6 ± 0.2

**Yeast Nitrogen Base**

Yeast nitrogen base (YNB) medium was prepared according to the direction given by the manufacture [Difco] and adjusted to pH 6.0 and sterilised by membrane filtration (0.45 μm). The medium was supplemented with 2.5% (w/v) glucose (pH 6.0) which was sterilised separately by autoclaving and the final medium was prepared by pipetting, under aseptic condition, 5 ml of YNB medium into 45 ml of 2.5% (w/v) glucose to give final medium of Yeast Nitrogen Base plus glucose (YNBG).

**Mycelial Medium (S.S.V.)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.0</td>
</tr>
<tr>
<td>Starch</td>
<td>1.0</td>
</tr>
<tr>
<td>((\text{NH}_4)\text{SO}_4)</td>
<td>2.5</td>
</tr>
<tr>
<td>(\text{KH}_2\text{PO}_4)</td>
<td>1.25</td>
</tr>
<tr>
<td>(\text{CaCl}_2)</td>
<td>0.10</td>
</tr>
<tr>
<td>(\text{MgSO}_4\cdot7\text{H}_2\text{O})</td>
<td>0.05</td>
</tr>
<tr>
<td>(\text{MgCl}_2\cdot6\text{H}_2\text{O})</td>
<td>0.16</td>
</tr>
<tr>
<td>Vitamin Solution*</td>
<td>10 ml/L</td>
</tr>
</tbody>
</table>

* Vitamin Solution

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>Pyridoxin</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>Thiamin HCl</td>
<td>2.0 mg</td>
</tr>
</tbody>
</table>
Meso-inositol 10.0 mg  
Distilled water 100 ml

Sterilise by membrane filtration (0.45 μm)

Hanks Balanced Salt Solution (HBSS)\(^3\)

<table>
<thead>
<tr>
<th>Part 1 (g/L)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium chloride</td>
<td>0.4</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>0.06</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.06</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>4.358</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Part 2 (g/L)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium chloride</td>
<td>0.14</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.10</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>0.10</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>3.642</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Part 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium bicarbonate solution 1.4%</td>
<td>14.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The contents of part 1 was dissolved in one liter of deionised water and sterilised by autoclaving (Stock solution 1). Correspondingly, the content of part 2 was dissolved in one liter of deionised water and sterilised by autoclaving (Stock solution 2). One hundred ml from stock solution 1 was added to 800 ml deionised water followed by 100 ml from stock solution 2. The mixture was sterilised by autoclaving for 15 minutes at 121°C. After cooling to room temperature, 25 ml from sterile sodium bicarbonate solution (part 3) was added aseptically.
Phosphate Buffered Saline (PBS)

Solution A

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>8.0</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.2</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>1.15</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.20</td>
</tr>
</tbody>
</table>

pH adjusted to 7.2 with 5N NaOH

Solution B

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium chloride</td>
<td>0.1</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>0.1</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

The contents of solution A was dissolved in 995 ml of de-ionised water and autoclaved for 10 min at 115°C. To make the complete PBS, 5 ml of sterilised solution B was added aseptically to 995 ml of solution A.

4. DETERMINATION OF MINIMUM INHIBITORY AND MINIMUM FUNGICIDAL CONCENTRATIONS (MIC & MFC):

The antifungal drugs used, with the exception of 5-FC, were dissolved in dimethylsulfoxide (DMSO). 5-FC was dissolved in YNBG. Octenidine and piritenidine were dissolved in sterile distilled water. All of these were sterilised by membrane filtration (0.45 μm) (Sartorius, GmbH, W.Germany).

An agar dilution method was used to determine the MIC for various Candida species used with the exception of 5-FC which was determined by tube-dilution method. Serial two-fold dilutions of the drugs ranging from 800 to 0.1 μg/ml were prepared in molten SDA, then poured and left to solidify. Plates were inoculated with an overnight cultures (10^7 cells/ml) of the organisms using a multipoint inoculator (Denley, U.K.). Plates were incubated at 37°C (Gallenkamp incubator, U.K.) for 48 hours, when the MIC value was recorded.
In some experiments, tube-dilution method was used to determine the MIC. Serial two-fold dilution of drugs under study were prepared in tubes containing 5 ml of SDB (Difco) except 5-FC where YNB supplemented with 0.15% asparagine and 1% dextrose was used. Tubes were inoculated with 0.02 ml containing $2 \times 10^6$ cells of fresh overnight (16-18 h, 37°C) broth culture. All tubes were then incubated at 37°C for 24 h where turbidity in the control tubes was visible, when the final readings were made. MIC values were noted in both cases as the lowest concentration showing no visible growth. One loopful of the broth tubes showing no visible growth was further subcultured on SDA plate to determine MFC. The plates were incubated at 37°C for 24 h and then checked for viability.

5. GROWTH STUDIES:

a. Optical Density

Growth rates were measured in the presence and absence of individual drugs. Candida spp. were inoculated into 25 ml of SDB and incubated overnight with orbital incubator [Gallenkamp] (160 rpm) at 37°C. The cells were centrifuged (4,200 rpm for 15 min) and resuspended in a small volume of fresh medium. This suspension ($10^5$ cells/ml) was used to inoculate four flasks of YNB supplemented with 2.5% (w/v) glucose medium (50 ml) and incubated as above. Drugs were introduced into the medium 2 h after inoculation, as cells entered log phase. Drugs were added at 0.0 (control), 1/4, 1/2, and 1x MIC. Samples were withdrawn at 60 min intervals, and the rate of growth of shake cultures was followed by determining the optical density at 420 nm (SP 6-500 Pye Unicam, Cambridge, U.K.).

Since all antifungal drugs used were dissolved in a minimal amount of DMSO (BDH Chemicals) with the exception of 5-FC which was dissolved in YNBBG, and octenidine and piritenidine which were dissolved in sterile distilled water, an equivalent amount of DMSO, YNBBG and distilled water was added to control flasks (without drugs) as well as to a control flask which contained media only.
b. Viable Cell Counts

Viable cell counts were monitored following treatments with 1/4 x MIC levels for each drug by pour plate technique. Serial 10-fold dilution in sterile physiological saline (SPS) (0.85% NaCl w/v) were prepared from cells treated with these drugs. Drugs were introduced into the SDB medium 2 h after inoculation. Samples were withdrawn at 60 min intervals and diluted in SPS. One ml samples of appropriate dilutions were then plated in triplicate on SDA. The plates were left to dry at room temperature and incubated at 37°C. Yeast colonies were counted by using colony counter (Gallenkamp colony counter, U.K.) after 48-72 h and the results were expressed as log viable counts per ml. Each experiment was carried out in triplicate and the results expressed as their mean.

6. EFFECTS OF ANTIFUNGAL DRUGS ON GERM - TUBE FORMATION:

*C.albicans* ATCC 10231 as well as *C.albicans* KCCC 14172 cells grown for 24 h at 37°C with and without drugs were washed 3 times with PBS, pH 7.2, by centrifugation. An inoculum (10^7 cells /ml ) from each preparation was added to tubes containing 10 ml calf serum (Gibco, Grand Island, N.Y.) and incubated in a shaking water bath at 37°C. At zero, 60, 120 and 180 min, samples were removed and added to an equal volume of 1% glutaraldehyde in PBS for fixation. The number of yeasts with germ-tubes were determined microscopically by counting 100 to 200 cells in each sample with a phase contrast microscope (200). A cell was considered to have formed a germ-tube when a narrow tube extended from the mother cell [at least 2 μm long]. Each experiment was carried out in triplicate and the results expressed as their mean ± SE.

7. EFFECT OF DRUGS ON BUDDING CELLS:

*C.albicans* ATCC 10231 and KCCC 14172 were maintained on YNBG for 48 h. The cells were harvested by centrifugation and washed 3 times with sterile distilled water (10 ml each). Yeast cells (10^7 cells/ml) were inoculated into flasks containing 20 ml of
Eagle's medium (which supports bud formation of yeast), with and without drugs. Flasks were incubated at 25°C on a rotary shaker at 200 rpm. Samples were withdrawn at intervals and scored for bud formation by counting the mean number of *C. albicans* forming buds in every 300 cells.

8. **EFFECT OF ANTIFUNGAL DRUGS ON MORPHOLOGY**:

a. Scanning Electron Microscopy (SEM)

*C. albicans* KCCC 14172 as well as *C. albicans* ATCC 10231 were grown in flasks containing 100 ml YNBG with and without drugs in a shake culture (160 rpm) at 37°C and for 24 h.

SEM was carried out using the technique of Ghannoum and Al-Khars (313). Glutaraldehyde (TAAB Laboratories, Reading, U.K.) was added to broth cultures to give a final concentration of 1.5% (v/v). After 2 min contact, the cells were removed by centrifugation at 4,200 rpm at 4°C, for 15 min and resuspended in 2 ml of glutaraldehyde (5% w/v) for 16 h at 4°C. The cells were then removed by centrifugation (4,200 rpm, for 15 min), washed 3 times with sodium cacodylate buffer, pH 7, dehydrated by passage through an ethanol series (50% to 100%) and resuspended in 100% ethanol to give the required density. One drop of this suspension was allowed to air-dry on a microscope cover slip and then dehydrated over CaCl₂ (anhydrous) under partial vacuum. The samples were then coated with gold-palladium (60:40) in a high vacuum unit (Hummer X sputter Coater, Technics Inc., Alexandria, VA) to obtain a coating of approximately 2nm thickness. These samples were examined in a Stereoscan Electron Microscopy (Novascan 30, W. Germany) at an angle of 45°, and operated at 15 kV with a resolution of 10 Å. Photographs were obtained using Polaroid film (Polaroid, 4x5 Land Film, image 9 x 11.5 cm, Type 55, U.S.A.).

b. Transmission Electron Microscopy (TEM)

*C. albicans* cells were prepared for electron microscopy according to the method of Ghannoum (321). Cells were fixed with 2.5%(v/v) glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.0,
at 4°C for 2 h. Cells were placed in freshly made 2% (w/v) potassium permanganate (KMnO₄) solution at 4°C for 2 h. The cells were centrifuged at 4,200 rpm for 15 min and placed in a fresh solution of KMnO₄ for 2 h.

The cells were washed five times with distilled water by repeated centrifugation. They were then placed in a solution containing 1% (w/v) potassium dichromate and 1% uranyl acetate for 2 h at 4°C. These were washed twice with distilled water and embedded in 2% (w/v) Bacto-agar (Difco), left to set and cut into small cubes (0.5 - 1.0 mm³) which were dehydrated through an ethanol series [50% to 100% (v/v)]. The 100% ethanol was replaced with propylene oxide (TAAB, Reading) twice for 20 min and sample was embedded in Epon (TAAB Laboratories, Reading) by graded impregnation. TAAB 812 permix kit hard was used: Hardener (1) Dodecenyl succinic anhydride (D.D.S.A.) 100 ml quantity, Hardener (2) Methyl-endomethyleneephthalic anhydride (M.N.A.) 50 ml quantity, and Accelerator Tri-dimethylamine-methylphenol (D.M.P.-30) in 5 ml quantity. 50 ml of M.N.A. was added to the 100 ml of D.D.S.A. and the contents of the ampoules of accelerator (5 ml) was added. The mixture was mixed thoroughly, and approximately 100 g of resin is ready for use. The mixture can be stored for about 3 months.

All samples were embedded in beam capsules (TAAB Laboratories, Reading), sectioned using Reichert-Jung Ultramicrotome (W. Germany) with a glass knives (angle 55°) and collected in a water trough using New 200 Athene grids. Sections were post-stained with uranyl acetate (dissolved in 70% ethanol) for 20 min and lead citrate (TAAB laboratories, Reading), (0.04%) in solution with sodium hydroxide, 0.1N, carbonate free for 5 min. Extensive surface washing was done, first with sodium hydroxide, carbonate free and then with distilled water. These were dried using millipore filters. Electron micrographs were recorded with JOEL 100 CX electron microscopy operated at 60 or 80 kV accelerating voltage.
9. ADHERENCE STUDIES:

a. Preparation of Buccal Epithelial Cells

Buccal epithelial cells (BECs) were collected from six healthy adult males 20-30 years old by gently rubbing the inside of their cheeks with sterile tongue depressor and suspending the cells in 5 ml HBSS. The donors had no signs or symptoms of oral thrush and had not taken antibiotics for at least 3 months.

Cells were washed twice with 10 ml HBSS and collected by centrifugation (1,200 rpm, 10 min), resuspended to a concentration of $2 \times 10^5$ cells/ml of HBSS as determined by hemacytometer count. These cells were then used to study the adhesion of Candida spp. to BEC following the exposure of yeasts to sub-MIC levels of various antifungal agents. Only freshly prepared samples of BECs were used in the adherence assay.

b. Preparation of Yeast Cells

Overnight cultures of C. albicans were grown at 37°C in 50 ml medium composed of YNB (Difco) supplemented with 2.5% (w/v) glucose. Flasks (100 ml) containing 50 ml of the same medium were inoculated with 1 ml of the overnight culture and grown for 24 h in a shaking water bath at 37°C. The cells were harvested by centrifugation (4,200 rpm at 4°C for 15 min) and washed twice with HBSS ready for the adherence assay. In some experiments, yeast cells were grown for 12 h (exponential phase) while others for 24 h (stationary phase).

To study the effect of drugs on the adherence of Candida spp. to BEC, yeast cells were inoculated at $10^5$ cells/ml into growth medium (YNBG) in the absence or presence of 1/4, 1/2, 1/8 and 1/16 x MIC level of each drug. Cultures were incubated in a rotary shaker (160 rpm) for 24 h at 37°C. Yeast cells were then harvested by centrifugation (4,200 rpm at 4°C for 15 min), washed twice with HBSS, and resuspended to concentration of $1 \times 10^7$ cells/ml of HBSS, as determined by hemacytometer count. These
cells were used in the adherence assay.

In other experiments, drugs were diluted into overnight cultures of yeast cells, grown in YNBG, to produce a final concentration of 1/4, 1/2, 1/8 and 1/16 x MIC levels of each drug. These suspensions were then incubated in a rotary shaker (160 rpm) at 37°C for an additional 60 min. Subsequently yeast cells were washed with HBSS by centrifugation and standardised to 1x10^7 cells/ml of HBSS, as determined by hemacytometer count.

The effect of combination of AMB and 5-FC on the adherence was monitored by growing *C. albicans* KCCC 14172 in the presence of either AMB or 5-FC each at 1/4 x MIC, or combination of two drugs both at 1/8 x MIC in shaking at 37°C for 24 h. Alternatively this combination was added to control yeast grown cells and incubated for 60 min at 37°C. In both cases, cells were collected by centrifugation and washed twice with HBSS and standardised to 1x10^7 cells/ml of assay medium.

The effect of cations on drugs action was studied by incorporating two cations [45mM MgCl₂ and 85mM KCl] in the assay medium (SPS instead of HBSS) containing standardised number of yeast (1x10^7 cells/ml of assay medium) in the absence or presence of 1/4 x MIC of AMB or NY. Cultures were shaken in dark at 37°C for 60 min, then harvested and washed twice with PBS and used in the adherence assay.

c. Effect of Culture Age on Adherence of Yeasts to BEC

Exponential phase of *C. albicans* KCCC 14172 cells were inoculated at 10^5/ml into YNBG with or without the drug-s. These were grown to exponential phase (12 h) or stationary phase (24 h) at 37°C in orbital shaker. Cells were harvested by centrifugation (4,200 rpm, at 4°C for 15 min), washed twice with 10 ml HBSS and standardised to a concentration of 1x10^7 cells/ml of assay medium as determined by hemacytometer count. The adherence capability of these cells was compared with that of control yeast cells grown without drugs.
d. Effect of Viability of Yeasts on Adherence to BEC

To determine whether Candida cell viability was essential for BEC attachment, *C. albicans* ATCC 10231 was grown in YNB supplemented with 2.5% (w/v) glucose at 37°C for 24 h. The cells were harvested by centrifugation (4,200 rpm at 4°C for 15 min) and washed twice with HBSS, resuspended in 10 ml of the assay medium. This suspension (5x10^9 cells/ml) was heat-killed as described previously by Maisch and Calderone (183) at 65°C for 2 h in a shaking water bath (GFL mbH, W.Germany). The heat-killed organisms were washed twice with HBSS and resuspended to concentration of 1x10^7 cells/ml of assay medium as determined by hemacytometer count. Cell viability was tested by plate count performed in triplicate on SDA plates. Agar plates were incubated at 37°C for 48-72 h and viability was checked.

The effect of Formalin-killed cells was monitored by suspending 5x10^9 cells/ml in 10 ml of 0.5% Formalin in saline (v/v). The suspension was held at 4°C for 18 h before the yeast cells were washed twice with HBSS (183). Yeast concentration was adjusted to 1x10^7 cells/ml of assay medium; viability was quantitated as described above.

Also *C. albicans* ATCC 10231 was washed with acetone for three times and suspended in 10 ml of acetone. This suspension was held at room temperature for 4 h. The acetone treated cells were washed twice with HBSS and resuspended to concentration of 1x10^7 cells/ml of HBSS. Viability was carried out as described above. The adherence capability of these cells was compared with that of control yeast cells. Each assay was carried out in triplicate and the results expressed as their means.

e. Effect of Germination of *C. albicans* on Adherence

To study the effect of germination of *C. albicans* KCCC 14172 on adherence to BECs, *C. albicans* cells were inoculated into flasks containing 50 ml of SDB and incubated in a shaking incubator at 37°C for 24 h. Cells were harvested by centrifugation (4,200 rpm, at 4°C for 15 min) and the pellet was
suspended in 200 ml of SDB and re-incubated for 18 h in a shaking water bath at 37°C. Cells were harvested by centrifugation and pellet was suspended in 500 ml of SSV medium (174). At zero time, 100 ml of culture was harvested and the pellet was washed three times with 10 ml HBSS and standardised to 1X10^7 cells/ml of assay medium. The culture in SSV medium was incubated at 37°C in a shaking water bath and at 30 min intervals, 100 ml was removed aseptically and harvested by centrifugation and pellet was washed three times with HBSS and standardised as described above. At least 200 cells were counted in the suspension by light microscopy at 400 x and the mean number of germinated and non-germinated cells adhering to every 100 BECs was determined. Each assay was carried out in duplicate and the results expressed as their mean ±SE.

f. Adherence Assay

The adhesion of Candida spp. to BEC was studied using a previously described technique (151,189,196,264,299). Briefly, 2 ml of BEC suspension (2x10^5 cells/ml) and yeast suspension (1x10^7 cells/ml) were mixed in a sterile screw-capped bottle (12x75 mm). The mixture was incubated in a shaking water bath at 37°C (160 rpm) for 2 h. After the incubation period, BECs were collected and washed through a 20 μm (pore size) filter (Retsch, Idar-Oberstein, W. Germany) to remove non-adhering yeast cells. The BECs on the filter were washed twice with 5 ml volumes of HBSS under continual (gentle) agitation and finally suspended in 5 ml of HBSS.

A drop of this suspension was mounted on a glass slide, air dried, heat fixed, and stained with Gram crystal violet for 1 min. The adherence was determined by light microscopy at 400 x by counting the number of yeast cells adhering to every BEC for 100 BEC, taken at random. Each assay was carried out in triplicate and on three different occasions, and the Student's t-test was used on the means of those 100 observations to evaluate the differences in the adherence values. A P-value of < 0.05 was considered significant.
10. Effect of sub-MIC Concentration of Antifungals on Extracellular Polymeric (EP) Material:

The effect of sub-MIC of various antifungal agents on the release of extracellular polymeric (EP) materials was studied by growing _C. albicans_ KCCC 14172 in YNBG in an orbital shaker (GallenKamp incubator, U.K.) at 37°C for 24 h. Batches of medium (one litre in three litre Erlenmeyer flasks) were inoculated with 50 ml of the overnight yeast culture. Control and test flasks, each containing 1/4 x MIC levels of various antifungal agents or a combination of AMB plus 5-FC (each at 1/8 x MIC), were incubated at 37°C for 24 h in an orbital shaker operating at 160 rpm. Following incubation, yeast cells were harvested by centrifugation (4,200 rpm at 4°C for 15 min) and culture supernatant was collected. The pellet was washed twice with sterile distilled water in pre-weighed centrifuge tubes. The tubes were then left to dry overnight at 75°C and the dry weight was determined. EP was isolated from culture supernatants by precipitation with acetone and according to the method of Douglas and co-workers (207,208).

Batches of 500 ml from culture supernatant was added to 1.5 volumes cold (4°C) acetone containing approximately 1g sodium acetate to aid precipitation. After 2 h at 4°C, the resultant precipitate was collected by centrifugation (Beckman Instruments, Inc., California) at 20,000 rpm for 30 min at 4°C. The pellet was suspended in 10-15 ml distilled water and any insoluble material was removed by centrifugation (4,200 rpm for 20 min at 4°C). Water-soluble polymer was recovered by acetone precipitation (3 volumes; 2 h at 4°C), washed with ether and evaporated to dryness in vacuo. The dried material was weighed and the yield was calculated as a percentage of the combined dry weight of the yeast cells and EP.

11. Analysis of EP Materials:

Qualitative analysis of EP preparations was done by descending paper chromatography on Whatman No.4 paper of acid
hydrolysates. EP (2 mg) was hydrolysed in 2 ml of 2N H$_2$SO$_4$ at 105°C for 3 h in small test tubes. Neutralisation was effected with BaCO$_3$; the BaSO$_4$ thus formed was filtered off. The filtrate was subjected to mechanical agitation with an ion exchange mixture [Dowex 1-X8 and Dowex 50-X4, 1:1 by volume] for 30 min to remove amino acids and other ionic material (322). Upon removal of the resin, the solution was evaporated to dryness at 25°C under reduced pressure, re-dissolved in a few drops of distilled water and subjected to descending paper chromatography using the solvent system butan-1-ol: ethyl acetate: pyridine: water [30:30:25:20 by volume] (322). This solvent system can separate galactose from both sucrose and glucose after 16 h. Hydrolysis products and standard sugars (mannose, glucose, galactose, and GLcNAc) were visualised with alkaline silver nitrate reagent which prepared as follows:

a) AgNO$_3$ saturated in water

Acetone

0.1 volume

20.0 volume

b) NaOH, 0.5 g dissolved in 5 ml water and diluted to 100 ml with ethanol

The paper was first dipped through the silver reagent and the acetone then blown off. When dry, it was dipped through the alkali and again the solvent evaporated by using hair drier. Spots begin to appear giving dark brown to black in less than 10 min. The brown back ground was dissolved out by immersing the paper in 2M ammonia (322).

For quantitative analysis, total carbohydrate was estimated spectrophotometrically according to the method of Yamamoto and Rouser (323) and Dubois et al. (324). A stock solution of 2% anthrone in 98% H$_2$SO$_4$ (10 ml) was prepared. This stock solution was added to 90 ml of 87.5% H$_2$SO$_4$ and mixed well. Four ml from this reagent was added to 1 ml of acid hydrolysis products, and the mixture was heated in a boiling water bath for 4 min. The mixture was placed in ice for 15 min to cool rapidly. The absorbance was measured at 625 nm (SP6-500 Pye Unicam, Cambridge, U.K.) with that of a reagent (4 ml anthrone acid and 1 ml water)
as blank. The amount of hexoses present were determined from a standard curve obtained with a pure standard of glucose and mannose.

The protein content was determined by the Lowry method. One ml of hydrolysis products was mixed well with 2.5 ml of copper alkali solution (50 ml of 4% Na$_2$CO$_3$ and 50 ml of 2N NaOH and 1 ml of 2% CuSO$_4$·5H$_2$O and 1 ml of 2% Na/K tartrate) and allowed to stand at room temperature for 10 min. 0.25 ml of folin solution (20 ml of phenol was mixed with 20 ml of distilled water) was added directly to the copper alkali solution and hydrolysis products and allowed to stand for 10 min at room temperature. The absorbance was measured at 660 nm (SP6-500 Pye Unicam, Cambridge, U.K.). The amount of protein was determined from a calibration curve obtained from bovine serum albumin as standard.

12. PROTECTION AGAINST C. albicans GI COLONISATION AND DISSEMINATION BY SACCHARIDES IN EXPERIMENTAL ANIMALS:

a. Effect of Saccharides on C. albicans Adherence to Intestinal Tissues

The adherence of C. albicans to murine intestinal disks in vitro was monitored using a modification of the technique suggested by Clancy and Savage (325). This involved obtaining disks of duodenal tissue from female mice (strain MFI) of an average weight of 20 g. These disks were exposed for 30 min in vitro to suspensions of blastospores of C. albicans, $10^8$ cells/ml of the assay medium (PBS). Three different strains of C. albicans were assayed: ATCC 10231, KCCC 14172 and KCCC 13878. The disks were then washed 3 times with sterile PBS (5 ml each) to remove non-adherent yeasts and homogenised by using a sterile Ystral x 10/20 glass homogeniser (Ystral GmbH, Dottingen, W.Germany). The number of adhering yeasts per tissue was determined by plating a portion of the homogenate using pour plate technique. Serial 10-fold dilutions in sterile PBS were prepared from these homogenates. One ml samples of appropriate dilutions were plated in triplicate in SDA (Difco) containing 50 mg/L
chloramphenicol (Sigma); the plates were left to dry and incubated at 37°C. Yeast colonies were counted after 48-72 h and results were expressed as the mean colony forming units (CFU) per tissue disk.

To evaluate the effect of Candida cell wall components on the adhesion of yeast to intestinal tissues, the following experiments were performed:

1. Inclusion of 2% (w/v) of test compounds [mannose, GlcNAc, and CSE] in the adhesion assay medium (PBS). These disks were exposed for 30 min to suspensions of blastospores of C. albicans, 10^8 cells/ml of the assay medium. No test compounds were added to the assay mixture in the case of controls.

2. Pre-treatment of duodenal disks with 2% (w/v) test compounds for 30 min. These disks were then incubated in the presence of C. albicans blastospores for another 30 min at 37°C in an orbital shaker and then assayed for adhesion. Control disks were pre-treated with sterile distilled water containing no test compound.

3. To test the effect of post-treatment, i.e. treatment with saccharides following exposure to Candida blastospores, disks were suspended in PBS containing 10^8 yeast/ml for 30 min at 37°C, in an orbital shaker. This was followed by washing twice with PBS to remove non-adherent yeasts. Disks were then resuspended for another 30 min in buffer containing 2% (w/v) test compound and re-incubated. Controls consisted of suspension of yeast-treated disks in PBS containing no test compound.

The number of attached C. albicans was then determined as mentioned above.

b. Inhibition of Candida Attachment to GI Tract and Systemic Spread by Saccharides in vivo

Infant mice, 5 to 6 days old, were used to study the possibility of preventing GI colonisation and dissemination by C. albicans by treating the animals with saccharides (251). White
mice (MFI strain) were used to establish a breeding colony in the animal house of the Department of Zoology, Kuwait University. Offspring of these animals were used in all experiments. Infant mice were used rather than adults because oral-intragastric inoculation of infant mice with C. albicans leads to GI and systemic candidosis (225,226,250,251). Adult mice are resistant to colonisation unless they are pre-treated with antibiotics (247).

For oral challenge of mice, an overnight culture of C. albicans KCCC 14172 was grown at 37°C in YNB (Difco) supplemented with 2.5% (w/v) glucose. Flasks containing 50 ml of the same medium were inoculated with 1 ml of the overnight culture and grown for 24 h in a shaking water bath at 37°C. The cells were harvested by centrifugation (4,200 rpm,10 min at 4°C), washed twice with sterile PBS, and resuspended to the estimated inoculum size. This estimate was based on repeated dilution counts of similarly prepared suspensions, and the actual challenge dose was determined by a dilution count at the time of challenge.

1. Animal preparation and inoculation

Two days prior to challenge with C. albicans, infant mice were treated with various saccharides by orally introducing 20 uL of a 2% (w/v) solution of either mannose, GLcNAc or CSE. Six hours prior to the challenge, infant mice were removed from their mothers. Immediately before inoculation a further 20 uL of the saccharides were given. The yeast inoculum, containing approximately 1 x 10^8 cells in 20 uL, was delivered orally by using Eppendorf tips. Treatment with saccharides was continued on a daily basis. Control animals were treated similarly with sterile distilled water. After challenge, the animals were returned to mothers without concern for parentage. To check if the timing of the saccharide inoculation are important to prevent C. albicans colonisation and dissemination, other experiments were carried out where a group of mice were given a saccharide dose 30 min prior to Candida inoculation. The influence of saccharide treatment post-Candida inoculation was also tested.
The dose-response effect for CSE was carried out with a single time point, 48 h. In this group the mice were treated with different concentrations of CSE two days prior to challenge with \textit{C.albicans}. CSE treatment was continued for a further 48 h when the mice were sacrificed and the yeast CFU determined for the whole intestine and the stomach.

2. Enumeration of organisms

The levels of \textit{C.albicans} in the GI tract and selected body organs were determined by quantitative dilution plate counting. Animals were sacrificed and immediately dissected under aseptic conditions. Body organs were removed to sterile Ystral x 10/ 20 glass homogenisers (Ystral GmbH, Gottingen, W.Germany), in the following order: both kidneys, spleen, liver, stomach and whole intestine. The stomach and whole intestine were then gently washed in 3 changes of sterile PBS to remove digesta and non-adherent organisms. Tissues were homogenised in 5 ml sterile PBS and serial 10-fold dilutions in sterile PBS were prepared from these homogenates. One ml samples of appropriate dilutions were plated in triplicate in SDA (Difco) containing chloramphenicol (50 mg/L); the plates were left to dry and incubated at 37°C. Yeast colonies were counted after 2-3 days and the results were expressed as the mean CFU per gram weight of tissue.

3. Electron microscopy

To monitor the effect of test compounds microscopically, separate disks for control and tests [saccharide treated] were prepared for SEM. The kidneys, liver and intestine of two infant mice were also removed following the infection for the same purpose. These were placed directly into 2% (w/v) glutaraldehyde in 0.1M cacodylate buffer and sliced (2mm x 10mm x 10mm) with a sharp razor blade, and washed in 3 changes of buffer, then fixed overnight in the same fixative and processed for SEM. Fixed tissues were rinsed 3 times, 20 min each, in cacodylate buffer, post-fixed with 1% OsO₄ for 45 to 60 min and then washed 3 times, 10 min each, with distilled water. Tissues were then placed
into liquid nitrogen to solidify where they were subsequently cracked into small pieces. Cracked tissues were then dehydrated by passage through an ethanol series (50% to 100%). Specimens were stuck on stubs and coated under vacuum with gold : palladium (60:40) in a Hummer X Sputter Coater (Technics Inc., Alexandria, VA). Prepared tissues were examined in a Novascan scanning electron microscope operated at 15 kV with a resolution of 100 Å. Photographs taken at different magnifications of several fields from each specimen by using Polaroid film (Polaroid type 55, U.S.A.).
CHAPTER SIX

RESULTS
RESULTS

1. Minimum Inhibitory and Minimum Fungicidal Concentrations (MIC and MFC):

The minimum inhibitory and minimum fungicidal concentrations for four antifungal agents and for octenidine and pirtenidine against each strain tested, as determined by the serial dilution method, are given in table 7. AMB was the most active agent (MIC of 0.5 µg ml\(^{-1}\)) followed by NY (MIC of 1.0 µg ml\(^{-1}\)), pirtenidine (MIC of 1.5 µg ml\(^{-1}\)), octenidine (MIC of 3.0 µg ml\(^{-1}\)), MN (MIC of 15.6 µg ml\(^{-1}\)) and the least active was 5-FC (MIC of 25.0 µg ml\(^{-1}\)). Minimal variation was observed in the sensitivity of C. albicans ATCC 10231 and C. albicans KCCC 14172 towards the drugs tested. More variation was observed in the sensitivity of C. tropicalis KCCC 13605, and C. kefyr KCCC 13709 towards the antifungal agents, octenidine and pirtenidine.

There was more variation in the fungicidal effects of the drugs and the values were between 5.0 µg ml\(^{-1}\) for AMB and 50.0 µg ml\(^{-1}\) for 5-FC. For the Candida strains MFC values for pirtenidine and octenidine were between 12.5 and 25.0 µg ml\(^{-1}\), respectively. (Table 7).
Table 7

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values (µg/ml) for antifungal agents and for octenidine and pirtenidine.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Amphotericin B</th>
<th>Nystatin</th>
<th>Miconazole</th>
<th>5-Fluorocytosine</th>
<th>Octenidine</th>
<th>Pirtenidine</th>
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<tr>
<td></td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
<td>MFC</td>
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<td>MFC</td>
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<tr>
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<td>5.0</td>
<td>1.0</td>
<td>7.5</td>
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<tr>
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<td>7.5</td>
<td>15.6</td>
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<tr>
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2. Effect of Drugs on Growth:

a. Measurement of Growth by Optical Density:

The effects of various MIC concentrations of antifungal drugs on the growth curves of Candida strains showed that in all cases growth inhibition was concentration-dependent. Figure 11 shows inhibition of C. albicans KCCC 14172 growth in liquid cultures by various antifungal drugs. All drugs were added at 1/4 their MIC level. The pattern of growth inhibition was MN > NY > 5-FC > AMB.

Figures 12 - 23 presents representative examples of both C. albicans strains ATCC 10231 and KCCC 14172 responses to growth in the presence of different concentrations of the antifungal drugs, octenidine and pirtenidine. All the drugs had an effect on growth of C. albicans strains at MIC value. The influence of all drugs on the growth was gradual and concentration-dependent, with higher concentrations causing a more marked inhibition than lower concentrations. Similarly, all the drugs retarded the growth of C. tropicalis KCCC 13605 and C. keyfr KCCC 13709 with an increase in inhibition with concentration [refer to Appendix 1].
Figure 11. Growth curves of *C. albicans* KCCC 14172 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). All drugs were added at 1/4 their MIC. The arrow represents time at which drugs were added, (●---●) control; (○---○) AMB; (▲---▲) 5-FC; (▵---▵) NY and (□---□) MN.
Figure 12. Growth curves of *C. albicans* ATCC 10231 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●--●); 1/4 x MIC (○---○); 1/2 x MIC (■---■) and 1 x MIC (□---□) of AMB.

Figure 13. Growth curves of *C. albicans* ATCC 10231 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●--●); 1/4 x MIC (○---○); 1/2 x MIC (■---■) and 1 x MIC (□---□) of NY.
Figure 12

Figure 13
Figure 14. Growth curves of *C. albicans* ATCC 10231 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●—●); 1/4 x MIC (O---O); 1/2 x MIC (■---■) and 1x MIC (□---□) of 5-FC.

Figure 15. Growth curves of *C. albicans* ATCC 10231 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●—●); 1/4 x MIC (O---O); 1/2 x MIC (■---■) and 1x MIC (□---□) of MN.
Figure 14

Figure 15
Figure 16. Growth curves of *C. albicans* ATCC 10231 grown at 37°C as a shake culture in YNB medium supplemented with 2.5 % glucose (w/v) containing 0.0 (●—●); 1/4 x MIC (○—○); 1/2 x MIC (△—△) and 1 x MIC (□—□) of octenidine.
Figure 17. Growth curves of *C. albicans* ATCC 10231 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v) containing 0.0 (●---●); 1/4x MIC (○---○); 1/2 x MIC (△---△) and 1x MIC (□---□) of pirtenidine.
Figure 18. Growth curves of *C. albicans* KCCC 14172 grown as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●—●); 1/4 x MIC (○—○); 1/2 x MIC (■—■) and 1x MIC (□—□) of AMB.

Figure 19. Growth curves of *C. albicans* KCCC 14172 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●—●); 1/4 x MIC (○—○); 1/2 x MIC (■—■) and 1x MIC (□—□) of NY.
Figure 18

Figure 19
Figure 20. Growth curves of *C. albicans* KCCC 14172 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●--●); 1/4x MIC (○--○); 1/2x MIC (■--■) and 1x MIC (□--□) of 5-FC.

Figure 21. Growth curves of *C. albicans* KCCC 14172 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●--●); 1/4x MIC (○--○); 1/2x MIC (■--■) and 1x MIC (□--□) of MN.
Figure 20

Figure 21
Figure 22. Growth curves of *C. albicans* KCCC 14172 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v) containing 0.0 (●--●); 1/4x MIC (○---○); 1/2 x MIC (△---△) and 1 x MIC (□---□) of octenidine.
Figure 23. Growth curves of *C. albicans* KCCC 14172 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v) containing 0.0 (●--●); 1/4 x MIC (○--○); 1/2 x MIC (△--△) and 1 x MIC (□--□) of pirtenidine.
b. Measurement of Growth by Viable Count:
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The effect of drugs in killing cells are further examined in Figure 24 with measurements of viable cell counts, rather than absorbance. Log viable cell count is plotted as a function of time after treatment with drugs. Drugs were added to growing cultures of *C. albicans* KCCC 14172 at 1/4 its MIC value. The pattern of viable counts inhibition was 5-FC > MN > octenidine > pirtenidine > NY > AMB. The observed differences in viable cell counts vs optical density measurement is expected, since absorbance measurements reflect changes in total numbers of live plus dead cells in the culture, while viable cell counts reflect only the living cells.
Figure 24. Log viable count versus time of *C. albicans* KCCC 14172 grown as a shake culture at 37°C in SDB containing 0.0 (●---●); AMB (✦---✦); MN (■---■); NY (◇---◇); 5-FC (○---○); octenidine (△---△) and pirtenisone (▲---▲). All drugs were added at 1/4 x MIC. The arrow represent time at which drugs were added.
3. Influence of Drugs on Germ-Tubes Induction:

Germ-tube formation in new born calf serum was significantly suppressed when either of two strains of *C. albicans* was incubated for 24 h in the presence of 1/4 x MIC levels of various antifungal agents. The pattern of germ-tubes inhibition was NY > AMB > MN > 5-FC (Figure 25 A and B).

Table 8 shows the effect of various concentrations of both octenidine and pirtenidine on the germ-tube formation of *C. albicans* KCCC 14172. Both drugs were able to inhibit germ-tube formation, with octenidine shows greater effect as compared with pirtenidine, where the percent reduction of germ-tube formation by 180 min was between 93% and 77.5% for octenidine and pirtenidine at 1x MIC level, respectively.

4. Effect of Octenidine and Pirtenidine on Budding of *C. albicans*:

Stationary phase cells of *C. albicans* KCCC 14172 and *C. albicans* ATCC 10231 exhibit about 15% and 18% budding cells at zero time respectively. Incubation of control cultures of these two yeasts in Eagle's medium brings about a considerable increases in the percentage of budding cells to 75% after 3 h for *C. albicans* KCCC 14172 and 65% for *C. albicans* ATCC 10231 (Figure 26 and 27). Incubation of cultures of these yeasts in Eagle's medium supplemented with various MIC levels shows that both pirtenidine and octenidine inhibit budding in both yeast species. Addition of octenidine and pirtenidine to *C. albicans* KCCC 14172 led to a decrease in the percentage of budding cells even after 1 h. In most cases effectiveness was dose-dependent, i.e. higher concentrations effected higher decreases in budding cells.
Figure 25. Effect of various antifungal agents (each at \(1/4 \times\) MIC) on the germ-tube formation of \(C.\) \textit{albicans} KCCC 14172 [A] and \(C.\) \textit{albicans} ATCC 10231 [B] grown in new born calf serum at \(37^\circ\)C.
Table 8

Effect of octenidline and piritenidline on the germ-tube formation of *C. albicans* KCCC 14172

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Control</th>
<th>Octenidline 1/4MIC</th>
<th>Octenidline 1/2MIC</th>
<th>Octenidline MIC</th>
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<td>6±1</td>
<td>74±3</td>
<td>30±4</td>
<td>22±3</td>
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</table>

*Mean number of yeast forming germ-tube ± SE
Figure 26. Effect of various concentrations of pirtenidine in the percentage of budding cells of *C. albicans* KCCC 14172 [A] and *C. albicans* ATCC 10231 [B] grown in Eagle's medium at 37°C.

Figure 27. Effect of various concentrations of octenidine on the percentage of budding cells of *C. albicans* KCCC 14172 [A] and *C. albicans* ATCC 10231 [B] grown in Eagle's medium at 37°C.
Figure 26

Figure 27
5. **Effect on Morphology**

a. **Scanning Electron Microscopy** [SEM]

The morphology of *C. albicans* in the presence and absence of various antifungal drugs was compared by SEM. Morphological variations observed upon the growth of *C. albicans* ATCC 10231 and *C. albicans* KCCC 14172 in the presence of sub-inhibitory concentration (1/4 x MIC) of various antifungal drugs are shown in plates 1 to 12. Control cells were generally smooth walled, spherical to elongated in shape (plates 1, 7 and 8). They were in yeast form showed evidence of budding. At variance with control cultures, cells grown in the presence of sub-inhibitory concentrations of various antifungal drugs showed cell distortion and partial collapse of the outer cell envelope. The extent of this damage varied with the antifungal used, with AMB and NY showing the most damage (plates 2, 3 and 7), while the least damage was shown by 5-FC (plates 5 and 7). Treatment of *C. albicans* KCCC 14172 and ATCC 10231 with a combination of AMB plus 5-FC (both at 1/8 x MIC) caused extensive damage and yielded 100% damaged cells (plate 6).

Morphological variations in the growth of both yeast strains in the presence of octenidine and pirtenidine are shown in plates 9 to 12. After 1 h of incubation of yeast cells with 1X MIC level of both drugs, some of the cells showed evidence of weakening in the cell envelope with indications of cell collapse in some cases. After 24 h of incubation with 1/4 X MIC, an extensive cell damage, lysis and collapse of the cell structure and extrusion of cellular contents (plates 9 and 11). Increasing the concentration of these agents (1x MIC) led to more drastic damage to the yeast with cell collapse, lysis and concurrent leakage of cytoplasmic material with eventual death (plates 10 and 12).
Plate 1. SEM of *C. albicans* ATCC 10231, grown as a shake culture in YNBG medium. Cells were grown for 24 hours at 37°C. Micrograph shows yeast form and budding cells. Magnification x 3500.

Plate 2. SEM of *C. albicans* ATCC 10231, grown as a shake culture in YNBG medium containing 1/4 x MIC of AMB. Cells were grown for 24 hours at 37°C. Micrograph shows cell collapse. Magnification x 3500.
Plate 3. SEM of *C. albicans* ATCC 10231, grown as a shake culture in YNBG medium containing 1/4 x MIC of NY. Cells were grown for 24 hours at 37°C. Micrographs shows cell damage. Magnification x 3500.

Plate 4. SEM of *C. albicans* ATCC 10231, grown as a shake culture in YNBG medium containing 1/4x MIC MN. Cells were grown for 24 hours at 37°C. Micrographs shows some cell damage. Magnification x 3500.
Plate 5. SEM of *C. albicans* ATCC 10231, grown as a shake culture in YNBG medium containing 1/4x MIC of 5-FC. Cells were grown for 24 hours at 37°C. Micrographs shows some damage and cells are spherical to elongated in shape. Magnification x 3500.

Plate 6. SEM of *C. albicans* ATCC 10231, grown as a shake culture in YNBG medium containing both AMB plus 5-FC each at 1/8 x MIC. Cells were grown for 24 hours at 37°C. Micrographs shows almost complete cell damage, loss of intact cells and irregular cell shapes. Magnification x 3500.
Plate 7. SEM of C. albicans KCCC 14172, grown as a shake culture in YNBG medium containing 0.0 [control] (A); AMB (B); NY (C); MN (D); and 5-FC (E) all at 1/4x MIC. Cells were grown for 24 hour at 37°C. Micrographs shows damage of the cells, with some cells shows elongation in shape. Magnification x 3500.
Plate 8. SEM of *C. albicans* KCCC 14172, grown as a shake culture in YNBG medium. Cells were grown for 24 hours at 37°C. Micrographs show yeast form and budding cells, spherical to elongated cells with smooth surface. Magnification x 3500.
Plate 9. SEM of *C. albicans* KCCC 14172, grown as a shake culture in YNBG medium containing 1/4 x MIC of octenidine. Cells were grown for 24 hours at 37°C. Micrographs show some swelling and collapse of cells. Magnification x 4800.

Plate 10. SEM of *C. albicans* KCCC 14172, grown as a shake culture in YNBG medium containing 1 x MIC of octenidine. Cells were grown for 24 hours at 37°C. Micrographs show cell collapse and the presence of cytoplasmic materials. Magnification x 3500.
Plate 11. SEM of *C. albicans* KCCC 14172, grown as a shake culture in YNBG medium containing 1/4 x MIC of pirtenidine. Cells were grown for 24 hours at 37°C. Micrographs shows cell collapse and cytoplasmic debris. Magnification x 4800.

Plate 12. SEM of *C. albicans* KCCC 14172, grown as a shake culture in YNBG medium containing 1 x MIC of pirtenidine. Cells were grown for 24 hours at 37°C. Micrographs shows complete lysis of cells and presence of cytoplasmic debris and irregular cell shape. Magnification x 4800.
b. Transmission Electron Microscopy [TEM] :
-----------------------------------------------

To determine whether differences in cell wall ultrastructure of various cell surface features could account for some of the differences in Candida adhesion to BECs and also to see the effect of these agents on ultrastructural variations, cells of C.albicans KCCC 14172 were examined by TEM. Control C.albicans cells showed typical budding cells with multi-layered cell wall and an outer electron dense layer with some floccular layer, smooth envelopes containing distinct intracytoplasmic organelles (plates 13 to 16). Incubation of C.albicans cells for 24 h in the presence of 1/2 x MIC level of either octenidine or piritenidine showed evidence of gross ultrastructure alterations (plates 17 to 22). Cells tended to adopt irregular shapes and the cell walls become irregular in structure with deformities in the layering structure and apparent loss of internal cohesion. The cell wall was altered with electron transparent areas appearing between it and the cytoplasm. Cell membranes lost their integrity and cytoplasmic contents coagulated , giving rise to electrone-dense and electron-thin regions (plates 17 to 22).
Plate 13. Electron micrograph of a section of *C. albicans* KCCC 14172, fixed with glutaraldehyde, potassium permanganate, and potassium dichromate. Cells were grown on YNBG medium for 24 hours at 37°C. Micrographs shows the cell wall, cell membrane, mitochondria, nuclear membrane and an outer electron dense layer. Note the presence of an outer electron dense layer (arrow). Magnification x 16,000.

Plate 14. Electron micrograph of a section of *C. albicans* KCCC 14172 fixed by glutaraldehyde, potassium permanganate, and potassium dichromate. Cells were grown on YNBG medium for 24 hours at 37°C. Micrograph shows the cell wall layers, cell membrane, mitochondria, nuclear membrane. Note the presence of an outer electron-dense layer (arrow). Magnification x 28,000.
Plate 15. Electron micrograph of a section of *C. albicans* KCCC 14172 fixed by glutaraldehyde, potassium permanganate, and potassium dichromate. Cells were grown on YNBG medium for 24 hours at 37°C. Micrograph shows the cell wall layers, cell membrane, mitochondria, nuclear membrane. Note the presence of an outer electron-dense layer (arrow). Magnification x 13,000.

Plate 16. Electron micrograph of a section of *C. albicans* KCCC 14172 fixed by glutaraldehyde, potassium permanganate, and potassium dichromate. Cells were grown on YNBG medium for 24 hours at 37°C. Micrograph shows the cell wall layers, cell membrane, mitochondria, nuclear membrane. Note the presence of an outer floccular layer (arrow). Magnification x 13,000.
Plate 17. Electron micrograph of a section of *C. albicans* KCCC 14172 fixed by glutaraldehyde, potassium permanganate, and potassium dichromate. Cells were grown on YNBG medium for 24 hours at 37°C in the presence of 1/2 x MIC of pirtenidine. Micrograph shows mother cell and the bud with the cell wall layers, cell membrane, mitochondria, nuclear membrane. Note the presence of an outer floccular layer (arrow). Magnification x 13,000.

Plate 18. Electron micrograph of a section of *C. albicans* KCCC 14172 fixed by glutaraldehyde, potassium permanganate, and potassium dichromate. Cells were grown on YNBG medium for 24 hours at 37°C in the presence of 1/2 x MIC of pirtenidine. Micrograph shows alterations in the cell wall and cytoplasmic disturbances, cell membrane, mitochondria, nuclear membrane. Note the presence of an outer floccular layer (arrow). Magnification x 13,000.
Plate 19. Electron micrograph of a section of *C. albicans* KCCC 14172 fixed by glutaraldehyde, potassium permanganate, and potassium dichromate. Cells were grown on YNBG medium for 24 hours at 37°C in the presence of 1/2 x MIC of piritenidine. Micrograph shows alterations in the cell wall and cytoplasmic disturbances, cell membrane, mitochondria, nuclear membrane. Note the presence of an outer floccular layer (arrow). Magnification x 13,000.

Plate 20. Electron micrograph of a section of *C. albicans* KCCC 14172 fixed by glutaraldehyde, potassium permanganate, and potassium dichromate. Cells were grown on YNBG medium for 24 hours at 37°C in the presence of 1/2 x MIC of octenidine. Micrograph shows alterations in the cell wall and cytoplasmic disturbances, cell membrane, mitochondria, nuclear membrane. Note the presence of an outer floccular layer (arrow). Magnification x 8,300.
Plate 21. Electron micrograph of a section of *C. albicans* KCCC 14172 fixed by glutaraldehyde, potassium permanganate, and potassium dichromate. Cells were grown on YNBG medium for 24 hours at 37°C in the presence of 1/2 x MIC of octenidine. Micrograph shows alterations in the cell wall and cytoplasmic disturbances, cell membrane, mitochondria, nuclear membrane. Note the presence of an outer floccular layer (arrow). Magnification x 16,000.

Plate 22. Electron micrograph of a section of *C. albicans* KCCC 14172 fixed by glutaraldehyde, potassium permanganate, and potassium dichromate. Cells were grown on YNBG medium for 24 hours at 37°C in the presence of 1/2 x MIC of octenidine. Micrograph shows alterations in the cell wall and cytoplasmic disturbances, cell membrane, mitochondria, nuclear membrane. Note the presence of an outer floccular layer (arrow). Magnification x 13,000.
6. Effect on Adherence

a. Effect of Antifungal Drugs on the adherence of Candida species to Buccal Epithelial Cells in vitro:

Treatment of *C. albicans* (two strains), *C. tropicalis* and *C. kefyr* with four different antifungal drugs affected the adherence of yeasts to BECs (Tables 9 to 12). In general, pre-treatment of *Candida* spp. with antifungals for a short period (1 h) has less effect on adhesion than pre-treatment for long period (24 h). Comparison of adherence inhibiting activity of antifungals used in this study (all at 1/4 x MIC) gave the following pattern of activity: NY > MN > 5-FC > AMB (Table 13). This pattern holds for yeasts pre-treated with antifungal drugs for a long period (24 h) and applied to the two *C. albicans* strains tested. However, a different pattern of activity emerged when yeast cells were pre-treated with antifungals for a short period (1 h) and gave the following pattern of activity: MN > 5-FC > AMB > NY (Table 13). A difference also existed between the two *Candida* strains tested with a 1 h pre-treatment. Table 14 shows the percent reduction of adherence of *Candida* spp. to BEC following pre-treatment with 1/8 x MIC of various antifungal agents, and gave the following pattern of activity for the two strains of *C. albicans* MN > 5-FC > AMB > NY.

The effect of pre-treatment of the yeast cells for a long (24 h) or a short (1 h) period in the presence of various concentrations of octenidine resulted in inhibition of adherence of *Candida* spp. to BEC [Table 15]. Long period exposure (24 h) reduced adherence of all four yeast isolates tested to between 36-55% of the control value using 1/2x MIC and between 24-40% using 1/4 x MIC (Table 15). With the shorter exposure period (1 h) 1/2 x MIC still produced significant inhibition with all four strains, although this was less than that achieved with longer pre-treatment. With 1/4 x MIC significant inhibition of adherence was observed with the two *C. albicans* strains but not with *C. tropicalis* or *C. kefyr* were no significant reduction on adherence was observed (Table 15).
A similar experiment was performed using pirtenidine (Table 16). This gave results which were qualitatively similar to those using octenidine although, in general, the former drug was less potent. Long period exposure reduced adherence of all four yeast isolates tested to between 39-43% of the control value when using 1/2 x MIC, and to between 23-29% using 1/4 x MIC of pirtenidine (Table 16). With the shorter exposure period (1 h), 1/2 x MIC still produced significant inhibition with all strains except with *C. kefyr* which gave no significant reduction on adherence. With 1/4 x MIC significant inhibition of adherence was observed with the two *C. albicans* strains but not with *C. tropicalis* or *C. kefyr*. In general, incubation of Candida spp. with octenidine or pirtenidine for a short period has less effect on adhesion than incubation for a long period.

The effect of pre-treatment of yeast, BECs or both for 30 min with octenidine and pirtenidine prior to assay is presented in Table 17. This shows that reduction in adherence can be achieved by pre-treatment of either partner. No significant differences could be found between pre-treatment of either cell type or between the two drugs (*P > 0.05*). Pre-treatment of both partners resulted in a greater reduction in adherence compared to pre-treatment of either partner. Pre-treatment of either *C. albicans* KCCC 14172 or BEC with 1/2 x MIC of octenidine for 30 min led to a reduction in adherence of this yeast to BEC to 28% and 29% of the control value, respectively. Pre-treatment of both cells led to the greater reduction in adherence (42% of the control value) compared to when the yeast or BECs were pre-treated (Table 17). Similar results were obtained when using pirtenidine but to a lesser extent (Table 17).

Figure 28 compares adherence abilities of stationary phase (24 h) and exponential phase (12 h) *C. albicans* KCCC 14172 and also compares the effects of pre-incubation with inclusion of octenidine and pirtenidine in the assay medium. No significant differences was observed in the adherence characteristics between control exponential and stationary *C. albicans* cells to BEC (*P > 0.05*) (Figure 28). Growing of *C. albicans* in the presence of 1/2 x MIC of pirtenidine for 12 h or 24 h resulted in
adherence blockage ($P < 0.001$). However, growth of \textit{C. albicans} in the presence of 1/2 x MIC of octenidine showed similar results. Inclusion of either octenidine or piritenidine (1/2 x MIC) in the assay medium caused similar inhibition of adherence but this was usually less than with pre-incubation (Figure 28).

\textbf{b. Effect of Combination of AMB and 5-FC on Adherence:}

Pre-treatment of \textit{C. albicans} with a combination of AMB and 5-FC, both at 1/8 x MIC, led to stronger adherence inhibition than that obtained for yeast pre-treated with either one alone at higher concentrations [1/4 x MIC ]( Table 18). Exposure to the combination for short period [1 h] resulted in almost, additive effect, while exposure for a long period [24 h] led to considerable reduction in adherence of \textit{Candida} as compared to exposure to each drug alone, but less than additive (Table 18).

Amphotericin B alone suppressed adherence from 20% (1h pre-treatment) to 39% (24 h pre-treatment) from the control value, and 5-FC suppressed adherence from 31% (1 h pre-treatment) to 40% (24 h pre-treatment). Combination of these two antifungal both at 1/8 x MIC suppressed the adherence of \textit{C. albicans} to BEC to 48% for short-term incubation and 53% for long-term incubation, and this was greater than either drug by itself (Table 18).
Table 9

Effect of Pre-Incubation of Various *Candida* spp. In Subinhibitory Concentrations of Amphotericin B on Adherence to Buccal Epithelial Cells.

Mean number of adherent yeasts per 100 epithelial cells
(1 h Pre-Incubation)

<table>
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<tr>
<th>Organisms</th>
<th>Control</th>
<th>1/4 MIC P</th>
<th>1/8 MIC P</th>
<th>1/16 MIC P</th>
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<td>(24)</td>
<td>(22)</td>
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Mean number of adherent yeasts per 100 epithelial cells
(24 h Pre-Incubation)

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Note: Values in parentheses are ± SE. *P* values compared with control. N.S, not significant.
Table 10

Effect of Pre-incubation of Various *Candida* spp. In Subinhibitory Concentrations of Nystatin on Adherence to Buccal Epithelial Cells.

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<td><em>C. albicans</em></td>
<td>507</td>
<td>106</td>
<td>&lt;0.001</td>
<td>304</td>
<td>&lt;0.001</td>
<td>347 &lt;0.05</td>
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</tr>
<tr>
<td>KCCC 14172</td>
<td>(33)</td>
<td>(13)</td>
<td></td>
<td>(25)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>377</td>
<td>217</td>
<td>&lt;0.001</td>
<td>262</td>
<td>&lt;0.001</td>
<td>295 &lt;0.05</td>
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</tr>
<tr>
<td>KCCC 13605</td>
<td>(21)</td>
<td>(16)</td>
<td></td>
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</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>246</td>
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<td>138</td>
<td>&lt;0.001</td>
<td>167 &lt;0.05</td>
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<tr>
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<td>(24)</td>
<td>(13)</td>
<td></td>
<td>(13)</td>
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<td></td>
</tr>
</tbody>
</table>

Note: Values in parentheses are ± SE. P values compared with control. N.S, not significant.
Effect of Pre-Incubation of Various *Candida* spp. In Subinhibitory Concentrations of Miconazole Nitrate on Adherence to Buccal Epithelial Cells.

### Mean number of adherent yeasts per 100 epithelial cells (1 h Pre-incubation)

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Control</th>
<th>1/4 MIC</th>
<th>1/8 MIC</th>
<th>1/16 MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 10231</td>
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<td>268</td>
<td>299</td>
<td>346</td>
</tr>
<tr>
<td></td>
<td>(20)</td>
<td>(24)</td>
<td>(22)</td>
<td>(23)</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>508</td>
<td>251</td>
<td>285</td>
<td>315</td>
</tr>
<tr>
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<td>(24)</td>
<td>(18)</td>
<td>(22)</td>
<td>(27)</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>363</td>
<td>241</td>
<td>276</td>
<td>294</td>
</tr>
<tr>
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<td>(29)</td>
<td>(14)</td>
<td>(18)</td>
<td>(18)</td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>263</td>
<td>169</td>
<td>201</td>
<td>222</td>
</tr>
<tr>
<td>KCCC 13709</td>
<td>(26)</td>
<td>(15)</td>
<td>(18)</td>
<td>(20)</td>
</tr>
</tbody>
</table>

Note: Values in parentheses are ± SE. P values compared with control. N.S, not significant.

### Mean number of adherent yeasts per 100 epithelial cells (24 h Pre-incubation)

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Control</th>
<th>1/4 MIC</th>
<th>1/8 MIC</th>
<th>1/16 MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
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</tr>
<tr>
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<td>515</td>
<td>235</td>
<td>265</td>
<td>304</td>
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<tr>
<td></td>
<td>(35)</td>
<td>(25)</td>
<td>(33)</td>
<td>(32)</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>520</td>
<td>198</td>
<td>234</td>
<td>286</td>
</tr>
<tr>
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<td>(31)</td>
<td>(17)</td>
<td>(15)</td>
<td>(25)</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>360</td>
<td>201</td>
<td>228</td>
<td>275</td>
</tr>
<tr>
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<td>(27)</td>
<td>(16)</td>
<td>(12)</td>
<td>(17)</td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>250</td>
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<td>187</td>
<td>207</td>
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<td>(22)</td>
<td>(16)</td>
<td>(23)</td>
<td>(20)</td>
</tr>
</tbody>
</table>

Note: Values in parentheses are ± SE. P values compared with control. N.S, not significant.
Table 12

Effect of Pre-Incubation of Various Candida spp. in Subinhibitory Concentrations of 5-Fluorocytosine on Adherence to Buccal Epithelial Cells.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Control</th>
<th>1/4 MIC</th>
<th>P</th>
<th>1/8 MIC</th>
<th>P</th>
<th>1/16 MIC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
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<td>269</td>
<td>&lt;0.001</td>
<td>343</td>
<td>&lt;0.001</td>
<td>362</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ATCC 10231</td>
<td>(22) (17)</td>
<td></td>
<td></td>
<td>(19)</td>
<td></td>
<td>(19)</td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
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<td>282</td>
<td>&lt;0.001</td>
<td>342</td>
<td>&lt;0.001</td>
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<td>N.S</td>
</tr>
<tr>
<td>KCCC 14172</td>
<td>(20) (15)</td>
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<td></td>
<td>(15)</td>
<td></td>
<td>(16)</td>
<td></td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>371</td>
<td>262</td>
<td>&lt;0.001</td>
<td>286</td>
<td>&lt;0.05</td>
<td>345</td>
<td>N.S</td>
</tr>
<tr>
<td>KCCC 13605</td>
<td>(15) (17)</td>
<td></td>
<td></td>
<td>(19)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. kefyr</td>
<td>300</td>
<td>230</td>
<td>&lt;0.05</td>
<td>274</td>
<td>N.S</td>
<td>286</td>
<td>N.S</td>
</tr>
<tr>
<td>KCCC 13709</td>
<td>(14) (13)</td>
<td></td>
<td></td>
<td>(14)</td>
<td></td>
<td>(13)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Control</th>
<th>1/4 MIC</th>
<th>P</th>
<th>1/8 MIC</th>
<th>P</th>
<th>1/16 MIC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>509</td>
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<td>312</td>
<td>&lt;0.001</td>
<td>360</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ATCC 10231</td>
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<td></td>
<td></td>
<td>(14)</td>
<td></td>
<td>(13)</td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
<td>524</td>
<td>284</td>
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<td>290</td>
<td>&lt;0.001</td>
<td>320</td>
<td>&lt;0.001</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>(16)</td>
<td></td>
<td>(16)</td>
<td></td>
</tr>
<tr>
<td>C. tropicalis</td>
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<td>232</td>
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<td>255</td>
<td>&lt;0.001</td>
<td>262</td>
<td>&lt;0.001</td>
</tr>
<tr>
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<td></td>
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<td>(14)</td>
<td></td>
</tr>
<tr>
<td>C. kefyr</td>
<td>285</td>
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<td>214</td>
<td>&lt;0.05</td>
<td>250</td>
<td>N.S</td>
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<td>(17)</td>
<td></td>
<td>(12)</td>
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</table>

Note: Values in parentheses are ± SE. P values compared with control. N.S, not significant.
Table 13

Percent Reduction of Adherence of *Candida* spp. to Buccal Epithelial Cells Following Pre-Incubation In 1/4 MIC of Antifungal Agents

<table>
<thead>
<tr>
<th>Antifungals</th>
<th>C. albicans ATCC 10231 (%)</th>
<th>C. albicans KCCC 14172 (%)</th>
<th>C. tropicalis KCCC 13605 (%)</th>
<th>C. kefyr KCCC 13709 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>39</td>
<td>28</td>
<td>44</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>41</td>
<td>44</td>
<td>50</td>
<td>34</td>
</tr>
<tr>
<td>Nystatin</td>
<td>33</td>
<td>44</td>
<td>32</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>71</td>
<td>79</td>
<td>42</td>
<td>50</td>
</tr>
<tr>
<td>Miconazole-nitrate</td>
<td>45</td>
<td>50</td>
<td>34</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>62</td>
<td>44</td>
<td>41</td>
</tr>
<tr>
<td>5-Fluorocytosine</td>
<td>40</td>
<td>44</td>
<td>29</td>
<td>23</td>
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<tr>
<td></td>
<td>44</td>
<td>46</td>
<td>32</td>
<td>34</td>
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</tbody>
</table>

133
Table 14

Percent Reduction of Adherence of *Candida* spp. to Buccal Epithelial Cells Following Pre-Incubation In 1/8 MIC of Antifungal Agents

<table>
<thead>
<tr>
<th>Antifungals</th>
<th>Pre-Incubation time</th>
<th>C. <em>albicans</em> ATCC 10231</th>
<th>C. <em>albicans</em> KCCC 14172</th>
<th>C. <em>tropicalis</em> KCCC 13605</th>
<th>C. <em>kefyr</em> KCCC 13709</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>1h</td>
<td>30</td>
<td>21</td>
<td>34</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>37</td>
<td>42</td>
<td>45</td>
<td>29</td>
</tr>
<tr>
<td>Nystatin</td>
<td>1h</td>
<td>24</td>
<td>36</td>
<td>27</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>36</td>
<td>40</td>
<td>31</td>
<td>44</td>
</tr>
<tr>
<td>Miconazole-nitrate</td>
<td>1h</td>
<td>38</td>
<td>44</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>49</td>
<td>55</td>
<td>37</td>
<td>25</td>
</tr>
<tr>
<td>5-Flucytosine</td>
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<td>23</td>
<td>32</td>
<td>23</td>
<td>9</td>
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<tr>
<td></td>
<td>24h</td>
<td>39</td>
<td>44</td>
<td>25</td>
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</table>
Table 15
Effect of Pre-incubation of Various *Candida* spp. In Subinhibitory Concentrations of Octenidine on Adherence to Buccal Epithelial Cells.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Control</th>
<th>1/2 MIC</th>
<th>P</th>
<th>Red.</th>
<th>1/4 MIC</th>
<th>P</th>
<th>Red.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
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</tr>
<tr>
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<td>551</td>
<td>338</td>
<td>&lt;0.001</td>
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<td>374</td>
<td>&lt;0.001</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>(36)</td>
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<td></td>
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<tr>
<td><em>C. albicans</em></td>
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<td></td>
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</tr>
<tr>
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<td>346</td>
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<td>(28)</td>
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<td></td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>KCCC 13605</td>
<td>336</td>
<td>222</td>
<td>&lt;0.001</td>
<td>34</td>
<td>290</td>
<td>N.S</td>
<td>-</td>
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<tr>
<td></td>
<td>(18)</td>
<td>(18)</td>
<td></td>
<td></td>
<td>(21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>KCCC 13709</td>
<td>276</td>
<td>202</td>
<td>&lt;0.05</td>
<td>27</td>
<td>217</td>
<td>N.S</td>
<td>-</td>
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<tr>
<td></td>
<td>(24)</td>
<td>(16)</td>
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<td></td>
<td>(18)</td>
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</tr>
</tbody>
</table>

Mean number of adherent yeasts per 100 epithelial cells ± SE
(1 h Pre-Incubation)

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Control</th>
<th>1/2 MIC</th>
<th>P</th>
<th>Red.</th>
<th>1/4 MIC</th>
<th>P</th>
<th>Red.</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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</tr>
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<td>608</td>
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<td>56</td>
<td>367</td>
<td>&lt;0.001</td>
<td>40</td>
</tr>
<tr>
<td></td>
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<td>(25)</td>
<td></td>
<td></td>
<td>(28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
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<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>KCCC 14172</td>
<td>607</td>
<td>332</td>
<td>&lt;0.001</td>
<td>45</td>
<td>362</td>
<td>&lt;0.001</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>(46)</td>
<td>(32)</td>
<td></td>
<td></td>
<td>(28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>KCCC 13605</td>
<td>374</td>
<td>171</td>
<td>&lt;0.001</td>
<td>54</td>
<td>285</td>
<td>&lt;0.05</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>(27)</td>
<td>(16)</td>
<td></td>
<td></td>
<td>(18)</td>
<td></td>
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</tr>
<tr>
<td><em>C. kefyr</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCCC 13709</td>
<td>284</td>
<td>181</td>
<td>&lt;0.001</td>
<td>36</td>
<td>199</td>
<td>&lt;0.001</td>
<td>30</td>
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<tr>
<td></td>
<td>(23)</td>
<td>(13)</td>
<td></td>
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<td>(16)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean number of adherent yeasts per 100 epithelial cells ± SE
(24 h Pre-Incubation)

Note: Values in parentheses are ± SE. *P* values compared with control. Red., percent reduction. N.S., not significant.
Table 16

Effect of Pre-Incubation of Various *Candida* spp. in Subinhibitory Concentrations of Pirtenidine on Adherence to Buccal Epithelial Cells.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Control</th>
<th>1/2 MIC</th>
<th>P</th>
<th>Red.</th>
<th>1/4 MIC</th>
<th>P</th>
<th>Red.</th>
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</thead>
<tbody>
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<td>415</td>
<td>296</td>
<td>&lt;0.001</td>
<td>28</td>
<td>334</td>
<td>&lt;0.05</td>
<td>20</td>
</tr>
<tr>
<td>ATCC 10231</td>
<td>(24)</td>
<td>(23)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>408</td>
<td>290</td>
<td>&lt;0.001</td>
<td>29</td>
<td>323</td>
<td>&lt;0.05</td>
<td>21</td>
</tr>
<tr>
<td>KCCC 14172</td>
<td>(27)</td>
<td>(20)</td>
<td></td>
<td></td>
<td>(22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>299</td>
<td>229</td>
<td>&lt;0.05</td>
<td>23</td>
<td>253</td>
<td>N.S</td>
<td>-</td>
</tr>
<tr>
<td>KCCC 13605</td>
<td>(19)</td>
<td>(17)</td>
<td></td>
<td></td>
<td>(20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>230</td>
<td>205</td>
<td>N.S</td>
<td>-</td>
<td>219</td>
<td>N.S</td>
<td>-</td>
</tr>
<tr>
<td>KCCC 13709</td>
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<td>(16)</td>
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<td>(15)</td>
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<td></td>
</tr>
</tbody>
</table>

Mean number of adherent yeasts per 100 epithelial cells ± SE (1 h Pre-Incubation)

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Control</th>
<th>1/2 MIC</th>
<th>P</th>
<th>Red.</th>
<th>1/4 MIC</th>
<th>P</th>
<th>Red.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>545</td>
<td>311</td>
<td>&lt;0.001</td>
<td>43</td>
<td>402</td>
<td>&lt;0.001</td>
<td>26</td>
</tr>
<tr>
<td>ATCC 10231</td>
<td>(26)</td>
<td>(21)</td>
<td></td>
<td></td>
<td>(24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>523</td>
<td>313</td>
<td>&lt;0.001</td>
<td>40</td>
<td>387</td>
<td>&lt;0.001</td>
<td>26</td>
</tr>
<tr>
<td>KCCC 14172</td>
<td>(29)</td>
<td>(25)</td>
<td></td>
<td></td>
<td>(30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>355</td>
<td>201</td>
<td>&lt;0.001</td>
<td>43</td>
<td>275</td>
<td>&lt;0.05</td>
<td>23</td>
</tr>
<tr>
<td>KCCC 13605</td>
<td>(26)</td>
<td>(17)</td>
<td></td>
<td></td>
<td>(20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>271</td>
<td>164</td>
<td>&lt;0.001</td>
<td>39</td>
<td>193</td>
<td>N.S</td>
<td>29</td>
</tr>
<tr>
<td>KCCC 13709</td>
<td>(22)</td>
<td>(16)</td>
<td></td>
<td></td>
<td>(18)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean number of adherent yeasts per 100 epithelial cells ± SE (24 h Pre-Incubation)

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Control</th>
<th>1/2 MIC</th>
<th>P</th>
<th>Red.</th>
<th>1/4 MIC</th>
<th>P</th>
<th>Red.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>555</td>
<td>311</td>
<td>&lt;0.001</td>
<td>43</td>
<td>402</td>
<td>&lt;0.001</td>
<td>26</td>
</tr>
<tr>
<td>ATCC 10231</td>
<td>(26)</td>
<td>(21)</td>
<td></td>
<td></td>
<td>(24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>523</td>
<td>313</td>
<td>&lt;0.001</td>
<td>40</td>
<td>387</td>
<td>&lt;0.001</td>
<td>26</td>
</tr>
<tr>
<td>KCCC 14172</td>
<td>(29)</td>
<td>(25)</td>
<td></td>
<td></td>
<td>(30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>355</td>
<td>201</td>
<td>&lt;0.001</td>
<td>43</td>
<td>275</td>
<td>&lt;0.05</td>
<td>23</td>
</tr>
<tr>
<td>KCCC 13605</td>
<td>(26)</td>
<td>(17)</td>
<td></td>
<td></td>
<td>(20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>271</td>
<td>164</td>
<td>&lt;0.001</td>
<td>39</td>
<td>193</td>
<td>N.S</td>
<td>29</td>
</tr>
<tr>
<td>KCCC 13709</td>
<td>(22)</td>
<td>(16)</td>
<td></td>
<td></td>
<td>(18)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Values in parentheses are ± SE. P value compared with control. Red., percent reduction. N.S, not significant.
Table 17

Effect of pre-treatment of yeast and buccal cells with Octenidine or Pirtenidine (1/2 X MIC) on the adherence of *C. albicans* KCCC 14172 to BEC.

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>Mean adherence (± SE)a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Octenidine (P, % Reduction)b</td>
</tr>
<tr>
<td>Yeast Epithelial cells</td>
<td></td>
</tr>
<tr>
<td>- - (control)</td>
<td>380±21</td>
</tr>
<tr>
<td>+ -</td>
<td>273±17 (&lt;0.001, 28)</td>
</tr>
<tr>
<td>- +</td>
<td>268±17 (&lt;0.001, 29)</td>
</tr>
<tr>
<td>+ +</td>
<td>220±19 (&lt;0.001, 42)</td>
</tr>
</tbody>
</table>

a Data are expressed as mean numbers of yeast adhering to 100 BEC.
b Probability values compared with control.
Figure 28. Effect of octenidine and pirtenidine on exponential and stationary phase yeast adherence to BEC. Exponential phase C. albicans KCCC 14172 were grown on YNBG medium with (1/2x MIC) and without octenidine or pirtenidine. These were grown to exponential phase (12 hours) or stationary phase (24 hours) at 37°C.

A. Yeast grown for 24 or 12 hours without drug.
B. Yeast grown for 24 or 12 hours in the presence of drug.
C. Yeast grown for 24 or 12 hours and drug included in the assay medium for 2 hours.
Table 18

Effect of Pre-incubation of *C. albicans* KCCC 14172 in a Combination of Amphotericin B plus 5-Fluorocytosine on Adherence to Buccal Epithelial Cells.

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>1h Pre-incubation</th>
<th>24 h Pre-incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yeast adhering to 100 BEC (mean ± SE)</td>
<td>P</td>
</tr>
<tr>
<td>Control</td>
<td>509±22</td>
<td></td>
</tr>
<tr>
<td>Amphotericin B (1/4 MIC)</td>
<td>407±16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5-Fluorocytosine (1/4 MIC)</td>
<td>352±16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Amphotericin B plus 5-Fluorocytosine (both at 1/8 MIC)</td>
<td>265±15</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Note: P values compared with control. Red., percent reduction from control values.
c. Effect of Cations Supplementation on Antifungals Inhibition of Adherence:

To determine whether supplementation of exogenous ions into growth medium reversed the effects of antifungal agents on adherence of *C. albicans* to BEC was examined. Potassium chloride (85 mM) or Magnesium chloride (45 mM) were added to the assay medium containing 1/4 x MIC level of AMB or NY and incubated for 60 min. The reduction of adherence was not reversed by these cation supplementation. No statistical difference in the adherence ability of *C. albicans* cells pre-treated with either AMB or NY only or with the supplementation of the assay medium with the cations in the presence of AMB or NY ( \( P > 0.05 \) ) [Table 19]. Candidal adherence was independent of additions of Mg Cl₂ or KCl to the assay mixture containing 1/4 x MIC of antifungal drugs (Table 19).
Table 19

Effect of supplementation of different cations on the adherence of *C. albicans* KCCC 14172 to BEC after growth for 60 min. in medium containing 1/4 MIC levels of Nystatin or Amphotericin B.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Yeast adhering to 100 BEC (mean ± SE)</th>
<th>P</th>
<th>Red.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>543a (28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NY</td>
<td>309 (28)</td>
<td>&lt;0.001</td>
<td>43</td>
</tr>
<tr>
<td>AMB</td>
<td>359 (28)</td>
<td>&lt;0.001</td>
<td>34</td>
</tr>
<tr>
<td>NY + 85mM KCl</td>
<td>317 (30)</td>
<td>&lt;0.001</td>
<td>41</td>
</tr>
<tr>
<td>NY + 45mM MgCl₂</td>
<td>329 (29)</td>
<td>&lt;0.001</td>
<td>39</td>
</tr>
<tr>
<td>AMB + 85mM KCl</td>
<td>376 (33)</td>
<td>&lt;0.001</td>
<td>31</td>
</tr>
<tr>
<td>AMB + 45mM MgCl₂</td>
<td>384 (31)</td>
<td>&lt;0.001</td>
<td>29</td>
</tr>
<tr>
<td>Control + 85mM KCl</td>
<td>525 (32)</td>
<td>N.S</td>
<td>-</td>
</tr>
<tr>
<td>Control + 45mM MgCl₂</td>
<td>514 (35)</td>
<td>N.S</td>
<td>-</td>
</tr>
</tbody>
</table>

a This value refers to the adherence of control *C. albicans* not treated with antifungal agents or cations.

Note: Values in parentheses are ± SE. P values compared with control. Red., percent reduction. N.S, not significant.
d. Effect of Viability of Yeasts on Adherence to BEC:

To determine whether blastospore viability was essential for adherence to BEC, *C. albicans* ATCC 10231 (5x10⁹ cells/ml) were killed with heat (65°C for 2 h) or with 0.5% formaldehyde (4°C for 18 h) or by washing the cells with absolute acetone. The ability of such cells to adhere was compared with that of viable cells. The results of this experiment are presented in Table 20. Blastospores retained their attachment capabilities, although to a lesser extent than control cells, even though they were killed by these treatments. The relative adherence of heat killed blastospores was 50%, formaldehyde killed blastospores 54% and for acetone killed blastospores 50% (Table 20).

e. Effect of Germination of *C. albicans* on Adherence to BEC:

Direct counts of the number of germinated and non-germinated forms of *C. albicans* KCC1 4172 in suspensions was compared (Table 21). The percentage of germinated forms increased with time e.g. at 120 min the germinated form was 92.5 ± 0.5%. The results in Table 22 shows the percentage of germinated forms attached to BEC. For example, at 60 min 44% of the attached fungi were germinated, compared with 56% of the attached fungi were non-germinated. At 120 min, 72% of adherent fungi were germinated compared with only 28% of non-germinated adherent fungi. These results indicate that germinated *C. albicans* attach selectively and to a greater extent than do non-germinated yeasts (Table 22).
Table 20

Effect of viability of *C. albicans* ATCC 10231 on adherence to BEC

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of adhering yeast per 100 BEC (Mean ± SE)</th>
<th>P</th>
<th>Relative adherence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>542±25</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Heat killed cells</td>
<td>271±23</td>
<td>&lt;0.001</td>
<td>50</td>
</tr>
<tr>
<td>0.5% formaldehyde killed cells</td>
<td>293±25</td>
<td>&lt;0.001</td>
<td>54</td>
</tr>
<tr>
<td>Acetone washed cells</td>
<td>270±26</td>
<td>&lt;0.001</td>
<td>50</td>
</tr>
</tbody>
</table>

*Expressed as (adherence of killed yeasts/adherence of viable yeasts) x 100. Values represent the mean of three experiments.
Table 21

Percentage of yeast and germinated forms of *C. albicans* KCCC 14172

<table>
<thead>
<tr>
<th>Fungl/ml</th>
<th>Time (minute)</th>
<th>% yeast&lt;sup&gt;a&lt;/sup&gt; (mean ± SE)</th>
<th>% germinated&lt;sup&gt;b&lt;/sup&gt; (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0</td>
<td>94 ±1</td>
<td>6 ±1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>73.5±3.5</td>
<td>26.5±3.5</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>44.4±0.5</td>
<td>55.5±0.5</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>20.5±2.5</td>
<td>79.5±2.5</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>7.5±0.5</td>
<td>92.5±0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells remained in HBSS during the development of germ-tubes.

<sup>b</sup> Cells were germinated in SSV medium.
Table 22

Adherence of nongerminated and germinated forms of *C. albicans* KCCC 14172 to BEC

<table>
<thead>
<tr>
<th>Fungl/ml</th>
<th>Time (minute)</th>
<th>Attached fungi per 100 BEC ± SE&lt;sup&gt;a&lt;/sup&gt; (% of total attached fungi)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nongerminated</td>
</tr>
<tr>
<td>5×10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0</td>
<td>267±24 (75)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>376±25 (65)</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>435±28 (56)</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>165±16 (30)</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>188±17 (28)</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Mean of triplicate determination ± S.E.

Table 23 shows that exposure of *C. albicans* KCCC 14172 to sub-inhibitory concentration of antifungal drugs led to an increase in the excretion of EP material from this yeast. These polymers were extracted according to the method of Douglas and co-workers (207,208). Similar to the results of these authors, chemical analysis of the polymer obtained in this study showed that they are mannoprotein in nature containing hexoses [85-90%], and protein [7-9%] (Table 23). Mannose was the major sugar, making about 85% of the total carbohydrate. Plate 23 shows descending paper chromatography of the EP acid hydrolysate materials showing mannose as the major sugar.

The loss of EP material under the influence of antifungal drugs gave the following pattern 5-FC > MN > AMB > NY. The loss of these material were mostly under the influence of a combination of AMB plus 5-FC both at 1/8 X MIC (Table 23). For the same weight, the percentages of hexoses extracted from the supernatant of yeasts cultured with various antifungal drugs were increased when compared to the control grown cells. In contrast, the percentages of protein present in the EP materials extracted were decreased. The only exception to this is the composition of EP materials from supernatants of yeasts grown in the presence of a combination of AMB plus 5-FC, where the opposite was observed.
Table 23

Yield of Extracellular Polymer (EP) Obtain from Culture Supernatants of C. albicans KCCC 14172 Following Growth With and Without Subinhibitory Concentration of Various Antifungal Agents Singly (1/4 MIC) or In Combination with Amphotericin B plus 5-Fluorocytosine (Both at 1/8 MIC)

<table>
<thead>
<tr>
<th>Antifungal Agents</th>
<th>Control</th>
<th>AMB</th>
<th>NY</th>
<th>MN</th>
<th>5-FC</th>
<th>AMB + 5-FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry weight(a)</td>
<td>3.2±0.26</td>
<td>2.0±0.03</td>
<td>2.8±0.05</td>
<td>2.3±0.08</td>
<td>1.2±0.02</td>
<td>0.42±0.001</td>
</tr>
<tr>
<td>Percentage yield of EP(b)</td>
<td>2.0±0.41</td>
<td>3.55±0.52</td>
<td>2.8±0.2</td>
<td>5.1±0.3</td>
<td>7.3±0.8</td>
<td>9.2±0.9</td>
</tr>
<tr>
<td>% Hexoses from EP(c)</td>
<td>90</td>
<td>91.5</td>
<td>92</td>
<td>98</td>
<td>98</td>
<td>80</td>
</tr>
<tr>
<td>Absolute value of hexoses(d)</td>
<td>58±3</td>
<td>65±5</td>
<td>75±4</td>
<td>117±10</td>
<td>87±6</td>
<td>31±1</td>
</tr>
<tr>
<td>% Proteins from EP(e)</td>
<td>10.0</td>
<td>8.5</td>
<td>7.8</td>
<td>1.6</td>
<td>1.9</td>
<td>20.1</td>
</tr>
<tr>
<td>Absolute value of proteins(f)</td>
<td>6.5±0.9</td>
<td>6.0±0.2</td>
<td>6.3±0.7</td>
<td>1.8±0.1</td>
<td>1.6±0.1</td>
<td>7.8±1.0</td>
</tr>
</tbody>
</table>

\(a\) Expressed in mg/ml of culture.
\(b\) E.P. expressed as % from yeast dry weight.
\(c\) Obtained by Dubois method and expressed as % from EP.
\(d\) Obtained by Dubois method, absolute values in µg/ml culture.
\(e\) Obtained by Lowry's method and expressed as % from EP.
\(f\) Obtained by Lowry's method and absolute value in µg/ml culture.
Plate 23. Descending paper chromatography on Whatman No. 4 paper of acid hydrolysates of EP materials developed in butan-1-ol:ethyl acetate:acetic acid:pyridine:water (30:30:5:25: 15, V/V). Hydrolysis products (B) were identified comparing their chromatographic behaviour with: (A), glucose; (B) mannose ;(C) sample extract ; and (D) galactose.
8. Light and Scanning Electron Microscopy of Yeast Adherence:

Plates 24 to 26 shows light micrographs of C. albicans cells adhering to BEC. In most cases, each yeast cells were found embedded in a shallow depression of the BEC surface. It was shown that each BEC has one or more attached C. albicans.

Effect of growth of C. albicans in the presence (1/4 x MIC) or absence of antifungal drugs on adherence to BEC has been studied by scanning electron microscopy. Plates 27 to 34 shows typical yeast cells adhering to BEC. It should be noted that the yeast cells are also embedded in a shallow depressions to BEC. These figures shows cell distortion and partial collapse of the outer cell envelope, but the ability of C. albicans to attach to BEC after growth in the presence of these drugs still exists.
Plate 24. Light microscopy of *C. albicans* KCCC 14172 cells adhering to BEC. Magnification x 500.

Plate 25. Light microscopy of *C. albicans* KCCC 14172 cells adhering to BEC. Note the yeast cells (arrow) are embedded in shallow depressions of the BEC. Magnification x 750.
Plate 26. Light microscopy of *C. albicans* KCCC 14172 cells adhering to BEC. Note the yeast cells (arrow) are embedded in shallow depressions of the BEC. Magnification x 750.

Plate 27. Scanning electron micrograph of *C. albicans* KCCC 14172 cells adhering to BEC. Cells were grown in YNBG medium for 24 hours at 37°C. Note the yeast cells (arrow) are embedded in shallow depressions of the BEC. Magnification x 3,500.
Plate 28. Scanning electron micrograph of *C. albicans* KCCC 14172 cells adhering to BEC. Cells were grown in YNBG medium for 24 hours at 37°C. Note the yeast cells (arrow) are embedded in shallow depressions of the BEC. Magnification x 3,500.

Plate 29. Scanning electron micrograph of *C. albicans* KCCC 14172 cells adhering to BEC. Cells were grown in YNBG medium containing 1/4 x MIC of AMB for 24 hours at 37°C. Note the yeast cells (arrow) are embedded in shallow depressions of the BEC. Magnification x 3,500.
Plate 30. Scanning electron micrograph of *C. albicans* KCCC 14172 cells adhering to BEC. Cells were grown in YNBG medium containing 1/4 x MIC of NY for 24 hours at 37°C. Note the yeast cells (arrow) are embedded in shallow depressions of the BEC. Magnification x 3,500.

Plate 31. Scanning electron micrograph of *C. albicans* KCCC 14172 cells adhering to BEC. Cells were grown in YNBG medium containing 1/4 x MIC of MN for 24 hours at 37°C. Note the yeast cells (arrow) are embedded in shallow depressions of the BEC. Magnification x 3,500.
Plate 32. Scanning electron micrograph of *C. albicans* KCCC 14172 cells adhering to BEC. Cells were grown in YNBG medium containing 1/4 x MIC of 5-FC for 24 hours at 37°C. Note the yeast cells (arrow) are embedded in shallow depressions of the BEC. Magnification x 3,500.

Plate 33. Scanning electron micrograph of *C. albicans* KCCC 14172 cells adhering to BEC. Cells were grown in YNBG medium containing 1/4 x MIC of octenidine for 24 hours at 37°C. Note the yeast cells (arrow) are embedded in shallow depressions of the BEC. Magnification x 3,500.
Plate 34. Scanning electron micrograph of *C. albicans* KCCC 14172 cells adhering to BEC. Cells were grown in YNNG medium containing 1/4 x MIC of pirtenidine for 24 hours at 37°C. Note the yeast cells (arrow) are embedded in shallow depressions of the BEC. Magnification x 3,500.
Three *C. albicans* strains (ATCC 10231, KCCC 14172 and KCCC 13878) were compared for their capacity to adhere to murine disks of duodenal tissue. A variation in the attachment of these strains was observed (Table 24). This difference was pronounced particularly between the laboratory strain (ATCC 10231) and the two clinical strains (KCCC 14172 and 13878), the latter showing higher adherence capabilities to murine disks (Table 24).

a. **Effect of Various Saccharides on the Adhesion of *C. albicans* to Intestinal Disks:**

Figure 29 shows the effect of various saccharides on the adhesion of *C. albicans KCCC 14172* to intestinal disks. Inclusion of all the three compounds tested (mannose, GLcNAc and CSE), individually, in the adhesion mixture led to blockage of adherence of *C. albicans* to intestinal tissues to varying degrees. CSE was the most active saccharides followed by GLcNAc, and mannose was the least active (Figure 29 A). Pre- and post-treatment of intestinal disk with 2% saccharides enhanced the inhibition of adherence with pre-treatment resulting in a higher magnitude of inhibition [Log mean CFU/ tissue disk pre-treated with 2% CSE was 6.0 while with post-treated was 6.2] (Figure 29 B and C).
Table 24
Attachment of three *Candida albicans* strains to intestinal disk.

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of tissue disks</th>
<th>Mean No. of adhering yeasts ± SD (x 10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em> ATCC 10231</td>
<td>8</td>
<td>26 ± 1</td>
</tr>
<tr>
<td><em>C. albicans</em> KCCC 14172</td>
<td>8</td>
<td>103 ± 6</td>
</tr>
<tr>
<td><em>C. albicans</em> KCCC 13878</td>
<td>8</td>
<td>91 ± 4</td>
</tr>
</tbody>
</table>
Figure 29. Effect of Candida cell wall components on the adhesion of yeast to intestinal disk:

A. Inclusion of test compounds (2% of mannose, NAG or CSE) in the adhesion assay. Controls consisted of assay with no test compound.

B. Post-treatment, i.e. treatment with saccharides following exposure to C. albicans, disks were suspended in PBS containing 10^8 cells/ml for 30 min at 37°C in an orbital shaker. The disks were washed twice with PBS and re-suspended for another 30 min in buffer containing 2% test compound and re-incubated. Controls consisted of Candida suspension in PBS with no test compound.

C. Pre-treatment of duodenal disks with 2% test compounds for 30 min and the disks were re-incubated in the presence of C. albicans blastospores for another 30 min at 37°C in an orbital shaker. Control disks were pre-treated with sterile distilled water containing no test compound.
b. Scanning Electron Microscopy of Intestinal Disks:

SEM was employed to study the association between *C. albicans* KCCC 14172 and the intestinal disks. Plate 35 is a SEM of an intestinal segment from an control tissues treated with *C. albicans* only. Yeast cells were observed to be attached directly to the caecal epithelium or were seen embedded in mucous material. The control intestinal tissues were found to have the most *Candida* in association with the mucosal surface. Compared to control tissues, intestinal disks pre-treated with 2% mannose, CSE and GLcNAc shows very few yeast cells were associated with the caecal epithelium (plates 36 and 37). It was found that *C. albicans* associated with the caecal mucosal surface indirectly by attachment to other adherent yeast. Finally, depressions in the epithelium were also observed under *Candida* cells (plate 36), possibly due to enzymic lysis, which may allow *C. albicans* to stabilise itself with the epithelium after the initial adhesion, while in plate 37 yeast cells can be seen in association with the epithelium, and other yeast cells can be seen attached to mucous material and other yeast cells.

The SEM results confirmed the effect of pre-treated intestinal tissues with 2% (w/v) saccharides, with CSE was the most active saccharide (plate 36), followed by GLcNAc (plate 37), and mannose was the least active.
Plate 35. SEM of intestinal tissues from control disk treated with PBS and incubated with *C. albicans* KCCC 14172 for 30 min at 37°C in an orbital shaker. Micrographs shows direct attachment of *C. albicans* to intestinal epithelium. Note that some yeast are attached to the surface while others are attached to adherent yeast. Magnification X 2175.
Plate 36. SEM of intestinal tissues from 2% CSE-treated disk incubated with *C. albicans* KCCC 14172 for 30 min at 37°C in an orbital shaker. Micrographs show direct attachment of *C. albicans* to intestinal epithelium. Note that some yeast are attached to the surface while others are attached to adherent yeast. The number of adherent yeast decline as compared to control disk. Magnification X 3,500.
Plate 37. SEM of intestinal tissues from 2% GLcNAc-treated disk incubated with *C. albicans* KCCC 14172 for 30 min at 37°C in an orbital shaker. Micrographs shows direct attachment of *C. albicans* to intestinal epithelium. Note that some yeast are attached to the surface while others are attached to adherent yeast and some yeast cells appear to be located in depression in the surface of the colonised tissue. The number of adherent yeast decline as compared to control disk. Magnification X 2,175.
10. **Effect of Saccharides on the Systemic Spread from the GI Tract to Selected Organs of Individual Infant Mice**:

After oral challenge with $10^8$ CFU/mouse, animals were sacrificed at various time during a 72-h period post-inoculation, and body organs of saccharide treated and non-treated control animals were scored for the presence or absence of *C. albicans* (Table 25). The intestines were almost uniformly infected at each of the five test periods employed. Viable *C. albicans* were recovered from all the stomach cultured irrespective of whether the animals were saccharide treated or not. The only exception was for animals treated with GLcNAc where the number of infected stomachs was 100% at 3 h post-inoculation but decline thereafter (at 72 h infected stomach was (66.6%).

*C. albicans* cells spread systemically from the gut to visceral organs in the control animals with the number of infected livers, spleens and kidneys highest at between 3 and 6 h post-inoculation. In contrast, systemic spread of *C. albicans* from the gut in the saccharide-treated animals was drastically reduced with no yeast recovery from the kidneys and spleens of GLcNAc and CSE-treated animals (Table 25). However, *C. albicans* was recovered from kidneys of mannose-treated animals at 3h and 6h post-inoculation and spleens were transiently colonised at 3 h post-inoculation only. Although the liver of most saccharide-treated animals showed infectivity, the percentage of livers positive for yeast was much lower than the control animals. This was particularly true for CSE-treated mice (Table 25).
Table 25

<table>
<thead>
<tr>
<th>Organ</th>
<th>Time post-infection</th>
<th>Control</th>
<th>2% Mannose</th>
<th>2% NAG(^a)</th>
<th>2% CSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidneys</td>
<td>3 h 45.5 (5/11)(^b)</td>
<td>33.3 (2/6)</td>
<td>0.0 (0/6)</td>
<td>0.0 (0/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 h 36.3 (4/11)</td>
<td>33.3 (2/6)</td>
<td>0.0 (0/6)</td>
<td>0.0 (0/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 h 36.3 (4/11)</td>
<td>0.0 (0/6)</td>
<td>0.0 (0/6)</td>
<td>0.0 (0/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48 h 27.2 (3/11)</td>
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<td>0.0 (0/6)</td>
<td>0.0 (0/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72 h 27.2 (3/11)</td>
<td>0.0 (0/6)</td>
<td>0.0 (0/6)</td>
<td>0.0 (0/6)</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>3 h 45.5 (5/11)</td>
<td>16.6 (1/6)</td>
<td>0.0 (0/6)</td>
<td>0.0 (0/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 h 45.5 (5/11)</td>
<td>0.0 (0/6)</td>
<td>0.0 (0/6)</td>
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</tr>
<tr>
<td></td>
<td>24 h 36.3 (4/11)</td>
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<td>0.0 (0/6)</td>
<td>0.0 (0/6)</td>
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</tr>
<tr>
<td></td>
<td>48 h 36.3 (4/11)</td>
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<td>0.0 (0/6)</td>
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</tr>
<tr>
<td></td>
<td>72 h 27.2 (3/11)</td>
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<td>0.0 (0/6)</td>
<td>0.0 (0/6)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>3 h 72.7 (8/11)</td>
<td>66.6 (4/6)</td>
<td>50 (3/6)</td>
<td>16.6 (1/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 h 72.7 (8/11)</td>
<td>50 (3/6)</td>
<td>50 (3/6)</td>
<td>16.6 (1/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 h 72.7 (8/11)</td>
<td>33.3 (2/6)</td>
<td>50 (3/6)</td>
<td>16.6 (1/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48 h 63.6 (7/11)</td>
<td>16.6 (1/6)</td>
<td>50 (3/6)</td>
<td>0.0 (0/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72 h 54.5 (6/11)</td>
<td>16.6 (1/6)</td>
<td>50 (3/6)</td>
<td>0.0 (0/6)</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>3 h 100 (11/11)</td>
<td>100 (6/6)</td>
<td>100 (6/6)</td>
<td>100 (6.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 h 100 (11/11)</td>
<td>100 (6/6)</td>
<td>83.3 (5/6)</td>
<td>100 (6.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 h 100 (11/11)</td>
<td>100 (6/6)</td>
<td>83.3 (5/6)</td>
<td>100 (6.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48 h 100 (11/11)</td>
<td>100 (6/6)</td>
<td>66.6 (4/6)</td>
<td>100 (6.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72 h 100 (11/11)</td>
<td>100 (6/6)</td>
<td>66.6 (4/6)</td>
<td>66.6 (4/6)</td>
<td></td>
</tr>
<tr>
<td>Whole intestine</td>
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<td>100 (6/6)</td>
<td>100 (6/6)</td>
<td>100 (6/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 h 100 (11/11)</td>
<td>100 (6/6)</td>
<td>100 (6/6)</td>
<td>100 (6/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 h 100 (11/11)</td>
<td>100 (6/6)</td>
<td>100 (6/6)</td>
<td>100 (6/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48 h 100 (11/11)</td>
<td>100 (6/6)</td>
<td>100 (6/6)</td>
<td>100 (6/6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72 h 100 (11/11)</td>
<td>100 (6/6)</td>
<td>100 (6/6)</td>
<td>100 (6/6)</td>
<td></td>
</tr>
</tbody>
</table>

\(a\) N-Acetylglucosamine

\(b\) Numbers in parentheses are number positive/total number examined.
a. Colonisation of the GI Tract and Visceral Organs:

Heavy colonisation of the GI tract and visceral organs of infant mice follows oral inoculation with *C. albicans*. The GI colonisation persists in survivors to an age at which the animals are normally resistant. Mice with persistent infections, therefore, were used to study the effects of saccharide-treatment on the colonisation and dissemination of *C. albicans*.

Treatment of mice with saccharides resulted in markedly reduced levels of *C. albicans* in the visceral organs (Table 26). This was particularly pronounced in the kidneys and spleen where *Candida* cells were not cultured from these organs of most of the animals. As shown in Table 26, control animals had high gut population which decreased with time. Similarly, saccharide-treated mice showed high GI tract colonisation. The levels of stomach colonisation by *C. albicans* in manna se and GLcNAc-treated animals were lower than that obtained in the control animals throughout the study period. Counts were between 2.5 fold to 22 fold lower than the control values. CSE-treated animals initially showed a slightly colonisation than the control, particularly at 6 h and 24 h (Table 26). This was followed by lower levels of colonisation at 48 h and 72 h (counts between 9-fold and 43-fold lower than in control animals). In general, the intestines of saccharide-treated animals had lower counts than the control animals at 3 h post-inoculation (between 2 fold and 3.7 fold lower than the control). At 6 h and 24 h post-inoculation the number of CFU in saccharide-treated animals were either similar to controls or slightly higher. This situation reversed thereafter (Table 26).

Treatment of mice with a single dose of 2% (w/v) solution of GLcNAc or CSE, 30 min prior to yeast inoculation, led to a reduction in the levels of *Candida* in the kidneys, spleen and liver (Table 27). In contrast, the transient colonisation of the stomach and intestine, exposed to the same treatment, increased significantly. Similar treatment with mannose gave inconsistent results (Table 27). Treatment with the saccharides post-*Candida* inoculation did not produce any significant differences in
transient colonization of the GI or systemic spread between the control and treated mice.

b. Dose-Response Effect of CSE in Both Whole Intestine and Stomach:

Concentration-dependence of the inhibitory activity of CSE was tested at a single time period (48 h). Figure 30 shows that inhibition by this saccharide derivative of transient yeast colonisation of the intestine and stomach of mice is dose-dependent, with higher concentrations resulting in lower colonisation. At 1% concentration, CSE inhibited yeast colonisation of the intestine only slightly (4% reduction as compared with the control), while inhibition of stomach colonisation was higher at this concentration (14% inhibition as compared to the control). However, no significant difference was observed in the inhibition level between 3% and 4% CSE solution. In both cases the inhibition was around 14% for the intestine and 17% for the stomach (Figure 30).
Table 26
Population levels of Candida albicans in the GI tract and visceral organs of untreated and saccharide-treated mice.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Time post-infection</th>
<th>Control</th>
<th>2% Mannose</th>
<th>2% NAG(^a)</th>
<th>2% CSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidneys</td>
<td>3 h</td>
<td>1.0(^b)</td>
<td>0.35</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>0.95</td>
<td>0.09</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>1.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>0.75</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>0.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Spleen</td>
<td>3 h</td>
<td>75</td>
<td>60</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>150</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>22.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>32.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>46</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Liver</td>
<td>3 h</td>
<td>2.9</td>
<td>1.25</td>
<td>0.125</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>0.6</td>
<td>0.8</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>1.65</td>
<td>0.125</td>
<td>0.08</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>2.1</td>
<td>0.075</td>
<td>0.08</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>4.1</td>
<td>0.2</td>
<td>0.065</td>
<td>ND</td>
</tr>
<tr>
<td>Stomach</td>
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<td>13500</td>
<td>4435</td>
<td>610</td>
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</tr>
<tr>
<td></td>
<td>6 h</td>
<td>6750</td>
<td>2000</td>
<td>478.5</td>
<td>9100</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>1015</td>
<td>1000</td>
<td>411.5</td>
<td>3140</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>1012.5</td>
<td>50</td>
<td>310</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>1010</td>
<td>67.5</td>
<td>208</td>
<td>23.5</td>
</tr>
<tr>
<td>Whole Intestine</td>
<td>3 h</td>
<td>3300</td>
<td>1470</td>
<td>1615</td>
<td>585</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>1115</td>
<td>1500</td>
<td>1216</td>
<td>477</td>
</tr>
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<td></td>
<td>24 h</td>
<td>160</td>
<td>477</td>
<td>915</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>165</td>
<td>33</td>
<td>27.5</td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>150</td>
<td>68</td>
<td>7</td>
<td>7.5</td>
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</table>

ND Not Detected

\(a\) N-Acetylglucosamine

\(b\) Average count from 6 animals x 10\(^4\)
Table 27

Population levels of *Candida albicans* in the GI tract and visceral organs of untreated and pre-treated mice with 20 μL saccharide.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Time post-infection</th>
<th>Control</th>
<th>2% Mannose</th>
<th>2% NAG</th>
<th>2% CSE</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>x 10^4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kidneys</strong></td>
<td>3 h</td>
<td>0.56^a</td>
<td>0.31</td>
<td>0.13</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>0.98</td>
<td>0.75</td>
<td>0.24</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>1.5</td>
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</tr>
<tr>
<td></td>
<td>48 h</td>
<td>0.66</td>
<td>2.1</td>
<td>0.33</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>0.41</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>3 h</td>
<td>26</td>
<td>5.3</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>75</td>
<td>22.3</td>
<td>2.3</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>24</td>
<td>36.5</td>
<td>3.8</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>26</td>
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<td>ND</td>
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<td></td>
<td>72 h</td>
<td>32</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
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<td>1.3</td>
<td>12</td>
<td>1.1</td>
<td>0.4</td>
</tr>
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<td></td>
<td>6 h</td>
<td>2.1</td>
<td>10.3</td>
<td>1.0</td>
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</tr>
<tr>
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<td>9.5</td>
<td>0.2</td>
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</tr>
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<td></td>
<td>48 h</td>
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<td>5.1</td>
<td>0.9</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>1.1</td>
<td>2.4</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Stomach</strong></td>
<td>3 h</td>
<td>417</td>
<td>4120</td>
<td>815</td>
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<td>536</td>
<td>99140</td>
<td>9414</td>
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<tr>
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<td>24 h</td>
<td>149</td>
<td>198</td>
<td>416</td>
<td>8482</td>
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<td>48 h</td>
<td>512</td>
<td>6437</td>
<td>901</td>
<td>7379</td>
</tr>
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<td>72 h</td>
<td>468</td>
<td>580</td>
<td>1460</td>
<td>5530</td>
</tr>
<tr>
<td><strong>Whole Intestine</strong></td>
<td>3 h</td>
<td>9734</td>
<td>7411</td>
<td>7699</td>
<td>5760</td>
</tr>
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<td>6 h</td>
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<td>4120</td>
<td>8603</td>
<td>3133</td>
</tr>
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<td></td>
<td>24 h</td>
<td>796</td>
<td>1952</td>
<td>3122</td>
<td>615</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>188</td>
<td>87</td>
<td>1297</td>
<td>595</td>
</tr>
<tr>
<td></td>
<td>72 h</td>
<td>168</td>
<td>5.8</td>
<td>25</td>
<td>377</td>
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</table>

ND Not Detected

a Average count from 6 animals x 10^4
Figure 30. Dose-response effect of CSE in both whole intestine and stomach of infant mice. The mice were treated with 0.0%, 1%, 2%, 3% and 4% of CSE two days prior to challenge orally with 20 uL of *C. albicans* KCCC 14172 suspension (10^8 cell/ml). CSE treatment was continued for a further 48 hours and the yeast CFU determined for both organs.
Figure 30
c. Scanning Electron Microscopy of Infant Mice:

SEM examination of kidney and liver mucosa of infant mice revealed that *C. albicans* could attach to the mucosal surfaces of these organs by some of the mechanisms observed in intestinal disks. Plates 38 to 41 shows SEM of un-treated and pre-treated mice with 20 uL saccharides. Examination of the mucosal surfaces of saccharide-treated animals challenged with *C. albicans* showed that *Candida* cells associated with the mucosa by several distinct mechanisms. Yeast cells were observed to be attached directly to the epithelium. Plate 38 shows SEM of an kidney segment from an un-treated saccharide mouse showing blastospores of *C. albicans* which is adhering to the mucosal surface. At 6 h after intragastric inoculation of pre-treated mice with 20 uL CSE, where no more blastospores are seen (Plate 39). In plate 40 control mouse liver showing hepatic cells and blastospores of *C. albicans* in association with the mucosal surfaces. Plate 41 shows SEM of mouse liver pre-treated with 20 uL of CSE showing hepatic cells only.

From these plates, it is clear that control mice (inoculated with *C. albicans* only) had higher *C. albicans* population and therefore heavy colonisation, while pre-treated mice with 20 uL of 2% (w/v) CSE and challenged with *C. albicans* shows lower population and therefore lower levels of colonisation.
Plate 38. Scanning electron micrograph of control mouse kidney. Note the location of the nuclei (N) and the presence of blastospore adhering to the tissues (arrow). Magnification x 2000.

Plate 39. Scanning electron micrograph of infant mouse kidney treated with 2% CSE and challenged with C.albicans. Micrographs shows the presence of blastospore (arrow) and location of the nuclei (N). Magnification x 1750.
Plate 40. Scanning electron micrograph of control infant mouse liver challenged with \textit{C. albicans}. Micrographs shows the presence of blastospore (arrow), hepatic cells and sinusoids (S). Magnification x 700.

Plate 41. Scanning electron micrograph of infant mouse liver treated with 2\% CSE and challenged with \textit{C. albicans}. Micrographs shows the presence of blastospore (arrow), hepatic cells and sinusoids (S). Magnification x 800.
CHAPTER SEVEN

DISCUSSION
Minimum inhibitory and minimum fungicidal concentrations for the antifungal drugs used in this study against Candida spp. were similar to those reported for other Candida spp. and for C. albicans strains (17). Most Candida isolates were inhibited in vitro by AMB concentrations less than 3 μg ml⁻¹, by NY concentrations of 1-25 μg ml⁻¹. In vitro, MN MICs for Candida isolates have been reported in the range of 0.1 to 25 μg ml⁻¹, while in most surveys 5-FC gave MICs that range from less than 1 to 10 μg ml⁻¹ (17,20,52,67,68,72,75). In this study, AMB was the most active agent [MIC 0.5 μg ml⁻¹] and the least active was 5-FC [25.0 μg ml⁻¹]. The results presented in this study clearly demonstrate that both octenidine and pirtenidine exhibit potent fungistatic and fungicidal activities against a number of Candida spp. (MIC was 3 and 1.5 μg ml⁻¹ for octenidine and pirtenidine, respectively). The two drugs octenidine and pirtenidine have recently been developed as antibacterial and/or antiplaque mouthwashes properties (178). All drugs had an effect on the growth of Candida spp. at MIC value. The influence of all drugs on the growth was concentration-dependent.

Cell viability assays gave different levels of inhibition when compared to optical density measurements. The pattern of viable cell counts inhibition was 5-FC > MN > octenidine > pirtenidine > NY > AMB. When growth rate was estimated by following the optical density at 420 nm, the pattern of growth inhibition was MN > NY > 5-FC > AMB > octenidine > pirtenidine. However, in cell viability assays SDB was used as a culture medium while YNB supplemented with 2.5% (w/v) glucose medium was used for optical density measurements. The observed differences in viable cell counts versus optical density measurement is expected, since the latter do not define whether the observed effects were caused by inhibition of growth, by death of cells, or by changes in cell morphology which resulted in altered scattering of light. It is known that changes in assay medium, inoculum size, nature of the solvent and diluent used to prepare
a drug dilution series, the cation concentration of the medium and incubation temperature will affect the apparent inhibitory level of drugs (17).

**Effect of Antifungal Drugs on Adherence of Candida spp. to BECs in vitro:**

Interest in the influence of antifungal agents on the adherence of Candida to ECs was heightened following reports showing that sub-inhibitory concentrations of clinically used antibiotics reduced adherence of bacteria to ECs both in vitro and in vivo (175, 280, 314, 315).

The effect of antifungal agents such as imidazoles, polyenes and 5-fluorocytosine, on the adherence of Candida spp. to ECs has been investigated. Sobel and Obedeanu (214) were the first to study the effect of sub-inhibitory concentrations of ketoconazole on the adherence of C. albicans to ECs. They showed that pre-incubation of stationary or logarithmic-phase of C. albicans in ketoconazole at concentrations of 0.002 to 0.1 μg/ml for 4 h at 37°C had no effect on adherence to VEC. However, this treatment was associated with a dystrophic morphology of the blastospores, extensive clumping and reduced germination resulting in fewer individual candidal blastospore directly attached to the cell membrane (214). The mechanism responsible for this reduction was attributed to an indirect effect on germination and clumping rather than a direct effect on adhesin synthesis or expression (214). Anderson and Odds (215) confirmed the reported reduction, brought about by ketoconazole, in the attachment of C. albicans to VEC. They showed that inclusion of high ketoconazole concentrations in an adherence assay led to a reduction in number of C. albicans germ-tubes adhering to ECs (215). Brenciaglia et al. (308) showed that pre-incubation of C. albicans with ketoconazole at MIC and sub-MIC (1/2 and 1/4 of MIC values) for 3 and 72 h did not affect either the adherence of yeast to BEC or germ-tube formation. Effects on adherence were observed only after 72 h and at the highest concentrations tested, above MIC values (308). Brenciaglia et al. showed that miconazole to have similar effects on adherence as those reported for ketoconazole.
However, Macura (309) showed ketoconazole and clotrimazole to be effective on inhibition the adherence of *C. albicans* to BEC both at therapeutic and sub-inhibitory doses. Pre-treatment of the yeast with drugs inhibited their adherence to BEC stronger than the addition of the drug to the test medium (309). Macura showed that clotrimazole proved to be less active as compared to ketoconazole.

Odds and Webster (310) studied the effect of imidazole derivatives [clotrimazole and ketoconazole] and triazole derivatives [fluconazole and itraconazole] on the adherence of *C. albicans* to VEC *in vitro*. Clotrimazole, itraconazole or ketoconazole had no effect on the adherence of *C. albicans*, regardless of whether the drugs were used to pre-treat the fungi or the VEC or were added to the fungus / vaginal cell mixture (310). These results contradict the findings of Sobel and Obedeanue (214) and Anderson and Odds (215) and Macura (309). The source of these disparities is almost certainly methodological (310). Vuddhakul et al. (311) reported a significant reduction of adherence of *C. albicans* to dacron fibre microcolumns occurred after 24h incubation with miconazole, econazole and ketoconazole. All three imidazoles caused significant dose-dependent inhibition of adherence (311). The effect of these azoles on germ-tube formation was not investigated by Macura (309) and Vuddhakul et al. (311).

Polyenes investigated for their effect on adherence are AMB and NY. Sub-MIC of AMB inhibited germination and *C. albicans* adherence to both serum- coated plastic surfaces and fibrin matrices (312). Brenciaglia et al. (308) showed that pre- incubation of *C. albicans* with either sub-MIC or above MIC levels of AMB significantly reduced the adherence of this yeast to BEC. McCourtie et al. (302) showed that exposure of denture acrylic strips to therapeutic concentrations of AMB *in vitro* effectively inhibits the adherence of *Candida* spp. to acrylic. Macura (309) showed that AMB revealed a strong adherence inhibiting action, some what weaker than that of 5-FC. Similar result was obtained by Vuddhakul et al. (311) and they reported that AMB is among the
most potent of the ten antifungal drugs tested for their ability to inhibit adherence of *C. albicans*.

Compared with AMB, NY is reported to have less effect on the inhibition of adhesion of *C. albicans* to ECs. Brenciaglia et al. (308) showed that pre-incubation of *C. albicans* with NY, at MIC and sub-MIC levels, did not have a significant effect on adherence of this yeast to BEC. However, upon exposure of the yeast to NY at above MIC concentrations gave low values in adherence (308). In contrast, Macura (309) showed that pre-treatment of *C. albicans* with NY (both at sub-inhibitory and therapeutic levels) inhibited adhesion considerably, although this polyene turned out to be the least effective compared to other antifungal agents tested by Macura (309). Vuddhakul et al. (311) reported that *Candida* adherence was significantly decreased after short-term incubation with NY. Natamycin, the third polyene antifungal used by Vuddhakul et al. had no inhibitory effects on *Candida* adherence (311). Maniar and Mavdikar (317) showed that *C. albicans* is less susceptible to natamycin in vitro than to other polyenes.

Results reported for the effect of 5-FC, a fluoropyrimidine, on the adhesion of *Candida* to ECs are contradictory. Brenciaglia et al. (308) showed that 5-FC interfered with yeast adherence only after long pre-treatment periods (72 h) and only at MIC, or above MIC values. Although, they showed that 5-FC did not interfere with adherence at sub-inhibitory concentrations, it influenced germination at all the concentrations tested particularly after 72 h exposure (308). In contrast to these findings, Macura (309) found 5-FC, together with ketoconazole, to be the most effective inhibitor of *C. albicans* adherence when compared to other antifungal agents tested in her study. Vuddhakul et al. (311) showed that 5-FC produced a significant inhibition of adherence after 18 h and 24 h incubation, but not after 2 h incubation. The combination of AMB and 5-FC showed synergism between these two drugs on *Candida* adherence than either drug by itself (311).
Other drugs tested for their role in inhibiting Candida adhesion include ICI 195,739 and amorolfin. ICI 195,739 is a bis-triazole and amorolfin is cis-4 [3-(p-1,1-dimethylpropyl)-phenyl]2- methyl propyl] 2,6-dimethyl- morpholine hydrochloride. Vuddhakul et al. (311) showed that Candida adhesion was inhibited after short-term incubation (2h) with ICI 195,739, while amorolfin produced significant inhibition of adherence after long-term incubation (18 and 24 h), but not after 2h (short term incubation). Both of these drugs demonstrated synergism of Candida adherence when combined with AMB. Vuddhakul et al. (311) suggested that the mechanism by which ICI 195,739 interfere with Candida adherence may be due to direct action on the fungal cell-membrane where the putative "adherence proteins" are located.

Sub-inhibitory concentrations of antifungal drugs used in this study significantly blocked, to varying degrees, the adherence of Candida spp. to BECs, depending on the drug used, its concentration, the susceptibility of the spp. to the drug, and incubation time with the drugs. Pre-incubation of C.albicans (two strains), C.tropicalis and C.kefyr with these drugs inhibited their adherence between 17% and 79% of the control value. Pre-treatment of yeast cells for a short period (1 h) had less effect on adherence than pre-treatment for a long period (24 h). Comparison of adherence-inhibiting activity of antifungal drugs gave the following pattern of activity: NY> MN > 5-FC > AMB. This pattern holds for yeasts pre-treated with 1/4 x MIC of antifungal drugs for a long period (24 h) and applied to the two C.albicans strains tested. However, a different pattern of activity emerged when yeast cells were pre-treated with antifungal drugs for a short period (1 h). Pre-treatment with 1/4x MIC levels of antifungal drugs for a short period gave the following pattern of activity for the two C.albicans strains tested: MN > 5-FC> NY > AMB. Furthermore, treating C.albicans with a combination of AMB plus 5-FC, both at 1/8 x MIC level, led to stronger adherence inhibition than that obtained for yeast pre-treated with either one alone at 1/4 x MIC (Table 18). Vuddhakul et al. (311) reported that incubation of C.albicans with a combination of AMB and miconazole led to a decrease in Candida
adherence and was greater than that after incubation with either drug by itself. Because polyenes changes the fungal cell membrane, these drugs might facilitate the entry into fungi of other antifungals, with different modes of action, such as 5-FC. The combined inhibitory effects of the two agents would then exceed the sum of their effects separately (17,311,326).

The results presented in this study on the reduction of adherence of yeast species to BECs in vitro are accorded with those reported by several workers (214,215,309,311), but they are at variance with the results of Brenciaglia et al. (308) and Odds and Webster (310). The source of these disparities is almost certainly methodological (176-179,310). Several methods have been adopted for quantification of *Candida* adherence (176-179). The first report on the measurement of *Candida* adherence to ECs adopted techniques previously developed for quantitating bacterial adherence (242). This technique consisted essentially of incubating yeast and ECs for a period of time, removing the non-adherent yeast cells by filtration and finally determining the number of adhering yeasts microscopically. The methods used today in various laboratories do not vary greatly from this approach (212,238).

Assays based on radiometric estimations are another approach developed for the measurement of *Candida* adhesion. These entail labelling of yeast cells by growing them in a medium containing $^{14}$C-glucose before incubating with the ECs (180). Adherence is then determined radiometrically. Although techniques based on radiolabelling are rapid and less laborious, they are by no means free of problems (178). Such techniques do not give any indication of direct/indirect adhesion. Numerous in vivo and in vitro models have been developed to quantify and characterised *Candida* adhesion to both living and inanimate surfaces. These models suffer from many methodological difficulties and drawbacks, which could be responsible for the variations in results coming out from different laboratories and even from the same laboratory (177,178,310). The disparity in results reported in literature emphasizes the great need for standardised adhesion assay (177,178).
Differences observed in the activity vs. adherence as a response to pre-treatment with different antifungal drugs are not unexpected, since the antifungal drugs that have been studied, do not share the same mode of action. The antifungal activity of polyenes is related to their binding to sterols, particularly ergosterol (20,21). This interaction results in the formation of pores in the fungal membrane altering its permeability and leading to loss of cytoplasmic materials and eventual death (42, 43). By binding with yeast membrane sterols, polyenes lead to a decrease in hydrophobicity of the fungi which probably contributes to the observed reduction in adherence following treatment with these drugs (326). A possible explanation of discrepancy of results between AMB and NY, even though they are structurally similar, could be the heterogeneity of the mechanisms of polyene action (17).

Azole antifungal agents are characterised by heterogeneity of action mechanisms (17). However, the mode of action shared by all azoles involves the inhibition of ergosterol synthesis by a selective interaction with cytochrome P-450-dependent 14α-demethylase (85-87,96). Another important effect which is common to most azoles is their ability to prevent or greatly perturb hyphal growth of *C. albicans* (17). These drugs interfere with germ-tube formation which may account for the reduction of adherence of *Candida* to ECs (86). Relevant to adherence is the effect of azoles on cell wall structure. Sub-inhibitory concentrations of azoles, too low to cause direct damage of the yeast membranes, can cause alterations in cell structure (67,76,77). These changes could be a consequence of the effect of azoles on the yeast cell membranes and cell wall where the adhesin/s are located (186,204,205,255,256).

5-Fluorocytosine affects both protein and DNA synthesis (56-59). It enters the fungal cell by the enzyme cytosine permease. Inside the fungal cell, 5-FC is deaminated by cytosine deaminase to 5-FU which, in turn, is phosphorylated to 5-fluorouridine monophosphate. The latter is phosphorylated further and incorporated into the yeast RNA with eventual disruption of protein synthesis (56-59). Inhibition of DNA synthesis is brought
about by the formation of 5-fluorodeoxyuridine monophosphate, an inhibitor of thymidylate synthetase (58). Of relevance to adherence are the changes reported by several workers in the proportion of cell wall polysaccharides as well as enlargement of cell volumes as a consequence of 5-FC action (59, 62, 63).

The results described in this study clearly show that both octenidine and pirtenidine interfere with the adherence of Candida spp. to BECs in vitro. This has been shown to be the case for both short and long term pre-incubation with the drugs, although longer term pre-incubation was generally more effective. No statistical differences in the adherence ability of different growth phases of C. albicans was observed in this study. Although stationary-phase yeast showed greater adherence than exponential phase cells (445 ± 21 compared to 398 ± 23 yeast adhering to 100 BECs, respectively), this was not high enough to be statistically significant (P > 0.05). This applied for both octenidine and pirtenidine. King et al. (180), however, reported that stationary-phase yeasts attached to VECs in greater numbers than exponential-phase organism, although prolonged incubation of the culture (for more than 24 h) did not significantly enhance adhesion. By contrast, Segal et al. (195) reported a higher rate of adhesion with exponential-phase yeast. Other workers (196) noted that no significant difference was observed in the adherence characteristics between control exponential and stationary C. albicans cells to BEC (P > 0.05). The reason for this difference is not immediately evident, although it may be attributed to the different growth media used by the different groups, cell surface hydrophobicity, phenotypic and strain-related differences between the above studies. Hazen et al. (197) showed that cell surface hydrophobicity of exponential-phase yeast cells was significantly lower than that of cells at the stationary-phase. These results may partly explain the greater adherence of stationary-phase cells than for exponential-phase cells. In addition, both King et al. (180) and Segal et al. (195) used VECs, whilst BECs were used by others (196). The effect of octenidine and pirtenidine on adherence of C. albicans as reported above persisted, irrespective of the growth phase.
Exposure of *C. albicans* KCCC 14172 to 1/4x MIC of AMB or NY in the presence of excess KCl or MgCl$_2$ did not restore the adherence capacity of treated yeasts to BECs. Similar result was reported by Nugent and Couchot (312). The antifungal activity of polyenes leads to an increase in membrane permeability, with a consequent leakage of K$^+$ and Mg$^{++}$ out of cells, with an eventual cell lysis and death (17). This interpretation was supported by the finding that addition of K$^+$ and Mg$^{++}$ ions at high concentrations to cultures of *C. albicans* protects the organisms against the inhibitory effects of candididin and AMB methyl ester [ Kerridge et al. (327)]. The addition of the protecting ions does not prevent the association of the antibiotic with the protoplast membrane but instead maintains the intracellular ionic concentrations in spite of the fact that protoplast membrane is made permeable by the polyene [Kerridge, 1985 (43)]. The results of medium supplementation with either K$^+$ or Mg$^{++}$ suggest that simple changes in the permeability do not explain effects of AMB or NY on *Candida* adherence.

The mechanism by which antifungal drugs interfere with *Candida* adherence is unclear, but may be due to their effects on various factors, which are known to influence the adhesion process either directly [damage to the cell wall or membrane] where the putative adherence proteins are located [Shepherd et al. 1987 (106)] and excretion of extracellular polymeric materials (106, 207, 208), or indirectly [such as effects on germination and viability (211, 213)]. The effects of antifungal drugs on these factors have been studied in detail here.

**Effect of *Candida* Viability on Adhesion to BECs:**

In this study, it was shown that viability may not be essential for candidal adherence to BECs *in vitro* since blastospores retained their attachment capabilities, although to a lesser extent than control cells, even though they were killed (Table 20). Kimura and Pearsall (198) reported that viable yeast cells adhere better than non-viable cells, and it was found that this was related to changes in the yeast [particularly to germination] rather than to cell viability. No differences were
observed in the adherence of viable and non-viable cells once germination had occurred (198). Maisch and Calderone (183) reported that heat- and formaldehyde- killed cells did not adhere as well as viable cells. Similar observation was reported by Samaranyake and co-workers (199,201). Work carried out subsequently by Lee and King (202) and Klotz et al. (182) stressed the fact that the method of fungal killing bore a relation to adherence capacity. There is an agreement that formaldehyde- and heat- killed C.albicans cells still adhere, albeit not necessarily as well as live cells (178, 198,201,202). Gentle heat killing of the yeast results in some loss of polysaccharides and, presumably, adhesin/s and thus decreased adherence (178). Killing of the yeast by heating at 63°C for 2 h and at 70°C for the same period will result in considerable damage to the outer surfaces of the yeast cells, with concomitant loss of adhesin/s and consequently poor adhesion. This damage with no doubt lead to either loss of or damage of fibrillar-floccular layers and will therefore affect the process of adherence.

Effect of Drugs on Germ-tube Formation and Budding of C.albicans:

Sub-inhibitory concentrations of antifungal drugs tested significantly suppressed germ-tube formation when either of two strains of C.albicans was incubated for 24 h in the presence of sub-MIC levels of these drugs. The pattern of germ-tube inhibition was NY = octenidine > AMB > MN > 5-FC = pirtenidine [Figure 25 and Table 8]. Both octenidine and pirtenidine were very effective inhibitors of budding in both C.albicans strain with octenidine being the more potent in this respect [Figure 26 and 27].

The relationship between germination and adherence has been considered by a number of investigators and the difference in adherability to ECs between yeasts and hyphal forms of C.albicans has been claimed by several authors (198,211-213,215,277). Kimura and Pearsall (198) found that C.albicans adhered to BECs better under conditions conducive to germ-tube formation. In a subsequent study, these workers gave evidence for a selectively
greater potential for adherence of \textit{C.\textit{albicans}} hyphae \cite{211}. Sandin et al. \cite{212} demonstrated that germinated \textit{C.\textit{albicans}} had significantly greater adherence to BECs than blastospores, with germ-tube being 50 times more adherent than yeast cells. Anderson and Odds \cite{215}, evaluating the differences in the adherence of different morphological forms of \textit{C.\textit{albicans}} in the terms of fungal biomass and surface areas, rather than cell numbers attached per EC, observed that hyphae attached significantly better than germ-tubes with yeast forms adhering the least. For \textit{C.\textit{albicans}}, there is general agreement that hyphal germ-tubes adhere to host surfaces better than yeast blastospores. The results presented in this study confirm these findings and show that germinated \textit{C.\textit{albicans}} attached selectively and to a greater extent than non-germinated yeast cells.

Sobel and Obedeanu \cite{214} and Anderson and Odds \cite{215} showed that exposure of \textit{C.\textit{albicans}} cells to sub-inhibitory concentrations of ketoconazole led to a change in shape of \textit{C.\textit{albicans}} with morphological dystrophy, formation of large aggregates and inhibition of germ-tube formation which indirectly reduced the attachment of yeast to human cells. Brenciaglia et al. \cite{308} showed that pre-incubation of this yeast with ketoconazole at MIC and sub-MIC levels for 3 and 72 h did not affect germ-tube formation. Sub-MIC or above MIC levels of AMB inhibited germination of \textit{C.\textit{albicans}} after different exposure times \cite{308}. Nugent and Couchot \cite{312} showed similar results and reported that sub-MIC of AMB inhibit germination as well as adherence of \textit{C.\textit{albicans}} to both serum-coated plastic surfaces and fibrin matrices. No significant effect on germination was reported for NY pre-treated \textit{C.\textit{albicans}} \cite{308}. These workers showed that 5-FC interfered with \textit{C.\textit{albicans}} germination at all the concentrations tested particularly after 72 h exposure. The results presented in this study clearly show that germination was significantly suppressed, again to a different extent depending on the antifungal drugs used and time of incubation.
Morphological variations observed in this study upon growth of *C. albicans* (two strains) in the presence of sub-inhibitory concentrations of various antifungal drugs gave almost similar effects. Control cells were generally smooth walled spherical to elongated in shape. They were in the yeast form showing evidence of budding. At variance with control cultures, cells grown in the presence of sub-MIC concentrations of various antifungal agents resulted in cell distortion and partial collapse of the outer cell envelope. The extent of this damage varied with the antifungal used, with AMB showing the most damage. Treatment of *C. albicans* with a combination of AMB plus 5-FC (each at 1/8 x MIC) caused extensive damage and yielded 100% damaged cells. In most cases octenidine appeared to be more potent than pirtenidine and the effects observed were dose-related. The alterations in the cell surface (rough) are most probably due to the leakage of intracellular materials and to a change in cell permeability, which is in agreement with the transmission ultrastructural observations showing electron thin areas. The electron thin areas are probably due to decrease in density, such decrease can occur only through loss of cytoplasmic material through the cytoplasmic membrane (17,178).

The morphological and ultrastructural changes observed upon growth of *C. albicans* in the presence of sub-inhibitory concentrations of various antifungal drugs are consistent with its mode of action. Polak and Wain (62) showed that the mean diameter of yeast forms of *C. albicans* cells grown at sub-inhibitory level of 5-FC was found to be increased in size, while the mycelial forms continues to extend. SEM examination of *C. albicans* yeast cells treated with 5-FC showed that it exhibited slightly folded surfaces as compared to smooth surfaces in un-treated cells (66). Arai et al. (63) reported that ultrastructural changes occur in both the nucleus and cell wall of *C. albicans* after 2 h of incubation with 5-FC, the nucleus is larger, while after 12 h the nucleus is further enlarged and translucent with filamentous components appearing in it while the cell wall became progressively thinner (63). Similarly SEM
examination of *C. albicans* showed morphological changes resulting from incubation of this yeast with clotrimazole (77), miconazole (17, 67), econazole (17, 67) and ketoconazole (72). In all cases the incubation of *C. albicans* or other yeasts at fungistatic concentrations resulted in excessive changes in the cell envelope, primarily in the plasma membrane and in the structure and organisation of the cellular organelles. Polyenes also showed morphological and ultrastructural changes upon their addition to yeasts (17).

The differences in adhesion of *C. albicans* treated with antifungal drugs to BECs noted in this study may be explained by the alterations in the morphology of the organism which affect the location and biochemical nature of the *Candida* adhesin/s. Ultrastructural evidence indicates that specific interaction between *Candida* and ECs is mediated by a floccular-fibrillar adhesin layer present on the outer surface of the yeast (132, 153, 176-179, 186, 205, 209). This layer has been repeatedly defined by electron microscopy (132, 186, 205, 209). This adhesin layer is reported, in some studies, as being unevenly distributed on the cell surface or localised only at an adhesive site (155, 205, 209), while in other studies it is reported to have an ordered alignment around the cell wall (132). In addition, it is interesting to note that the degree of development of this outermost layer is related to the adherability; cells with thicker layers being more adherent as compared with cells with thinner layers (176-179, 186). The ultrastructural results of this study show that cells of *C. albicans* grown in media containing 1/2 X MIC of either octenidine or pirtenidine yielded cells that possessed the thinnest cell wall with the fewest apparent layers.

It is quite clear that this fibrillar-floccular layer may indeed mediate *Candida* adhesion to surfaces. The chemical nature of *Candida* adhesin/s is unknown with some reports proposing that *C. albicans* produce more than one adhesin (151, 176-179, 195, 209, 212, 224, 277). Segal and co-workers (195, 224, 279) have suggested that cell wall chitin, which is located at the innermost portion of the cell wall of *C. albicans* (106, 130, 153, 160), may serve as
the adhesive component. Other workers (151,264) gave experimetal evidence for the involvement of lipids in adherence of Candida spp. to BECs. Mannan or mannoprotein has been claimed by several investigators to serve as candidal adhesin (204-207,212, 223). Tronchin et al. (277) gave evidence for the presence of four specific proteins involved in C.albicans germ-tube adherence to plastic and these results clearly demonstrate that protein portion of the mannoprotein layer influences adhesion of Candida.

Effect of Antifungal Agents on the Release of Extracellular Polymeric Materials from C.albicans:

Exposure of C.albicans to sub-inhibitory concentrations of various antifungal agents led to an accumulation of extracellular polymeric (EP) materials in the culture supernatant. The extent of accumulation was again drug dependent. EP materials were extracted according to the method of Douglas and co-workers (207, 208). Similar to the results of these authors, chemical analysis of the EP materials obtained in this study showed that they are mannoprotein in nature. Mannose was the major sugar, making about 85% of the total carbohydrate. The loss of EP was mostly under the influence of AMB plus 5-FC combination followed by 5-FC > MN > AMB > NY. For the same weight, the percentages of hexoses extracted from the supernatant of yeast cultured with various antifungal agents were increased when compared to the control grown cells. In contrast, the percentages of protein present in the EP materials extracted were decreased. The only exception of this is the composition of EP from supernatants of yeasts grown in the presence of a combination of AMB plus 5-FC, where the opposite was observed.

It has been shown that sub-inhibitory concentrations of antibiotics selectively interfere with adherence properties of micro-organisms in various ways. They can inhibit the expression of fimbriae and the synthesis of certain excreted and non-excreted components of bacteria, and they may also cause the release of constituents from the cell such as lipoteichoic
acid [LTA], proteins, carbohydrates and others, which may act as bacterial adhesin (175, 280,314,315).

The work by Al-Bassam et al. (316) showed that sub-inhibitory levels of AMB led to *C. albicans* cell wall modification which involved peptidomannans. These components have been suggested by Douglas and co-workers (207,208) to be responsible for adhesion of *C. albicans* to ECs and acrylic strips. Al-Bassam et al. reported that growth of *C. albicans* in the presence of sub-MIC of AMB reduced the level of peptidomannans. This reduction was mainly due to a fall in the absolute and relative values of mannans. The treatment of *C. albicans* with tunicamycin, an antibiotic that inhibits protein glycosylation and, at low concentrations, inhibits mannoproteins synthesis but not chitin or glucan synthesis, interfered with the adherence of this yeast (179). Douglas and McCourtie (274) reported that the addition of tunicamycin, at the early stationary phase, inhibited the formation of the fibrillar layer with a consequential decrease in adherence to BEC. Results presented in this study clearly show that sub-inhibitory concentrations of various antifungal agents stimulate the excretion of EP materials known to mediate adherence of *C. albicans* to acrylic strips and ECs (207, 208). Douglas and co-workers provided evidence that protein portion of the mannoprotein adhesin is more important than the carbohydrate moiety in mediating attachment to BECs. This was confirmed by subsequent report by Tronchin et al. (277). Evidence to support the role of proteins as *Candida* adhesins comes from experiments showing that *C. albicans* cell adherence decrease following exposure to heat or various proteolytic enzymes (179,200,202,276). The results reported in this study show that growth of *C. albicans* in the presence of a combination of AMB plus 5-FC, both at 1/8 MIC, led to an increase in the percentage of protein obtained from culture supernatant. This increase may explain the reduction on adherence of this yeast to BECs than that obtained for yeast pre-treatment with either one alone at higher concentration (1/4 MIC), and also confirm the reported results that protein portion, in collaboration with the polysaccharide moiety of mannoprotein layer influences *Candida* adhesion to surfaces.
Adherence of *C. albicans* strains to Intestinal Disks:

Prior to *in vivo* experimentations, the attachment of *C. albicans* to murine intestinal tissues *in vitro* using a modification of the technique suggested by Clancy and Savage (325) was investigated. This represented an intermediate stage between *in vitro* experimentation using ECs and *in vivo* animal models. Three different strains were assayed: *C. albicans* ATCC 10231 (a laboratory strain), KCCC 14172 and KCCC 13878 (clinical strains). The results indicate that a variation in the attachment of different strains to murine intestinal tissues exist. This difference was pronounced particularly between the laboratory strain and the two clinical strains. Segal *et al.* (187) indicated that isolates of *C. albicans* from patients with vaginitis were significantly more adherent than isolates from asymptomatic carries. Kearns *et al.* (185) detected small differences in adherence between four laboratory strains of *C. albicans* that differed in virulence for mice, and between three pairs of minimally subcultured isolates from cases of oral thrush and from the mouths of healthy donors. McCourtie and Douglas (186) reported similar variation on adherence of strains following growth in the presence of low or high concentrations of sugars.

To evaluate the effect of *Candida* cell wall components (CSE, GLcNAc, and mannose) known to block adherence of yeast to ECs *in vitro* (183,195,202,291,212,223), on the adhesion of yeast to intestinal tissue, different experiments were performed. Inclusion of either mannose, GLcNAc or CSE, individually, in the adhesion assay mixture led to blockage of adherence of *C. albicans* to intestinal tissues to varying degrees with the CSE being the most active. Furthermore, pre-or post-treatment of duodenal disks with saccharides enhanced the inhibition of *Candida* adherence. The SEM results shown in this study confirm the effect of *Candida* cell wall components, CSE being the most active inhibitor. These results agree with the findings of Segal and Savage (224) who showed inhibition of *Candida* adherence to intestinal tissues under the influence of CSE. These authors did not, however, test the influence of either GLcNAc or mannose. Sandovsky-Losica and
Segal (328) showed that CSE inhibited the adhesion of C.albicans to GI tissues from both irradiated and non-irradiated mice by 75-85%. They also reported that adherence of C.albicans to all parts of the GI mucosa in irradiated mice was increased as compared to non-irradiated mice (328).

Mehentee and Hay (329) studied the in vitro adherence of C. albicans to murine GI mucosal surfaces in the presence of sub-inhibitory concentrations of AMB, ketoconazole and itraconazole. They showed that each antifungal drug had a significant ability to reduce the adherence of C.albicans to gastric and jejunal mucosa. These authors showed that the effects were influenced by the type of mucosal surface and its cellular arrangement and the concentration of the antifungal drug used (329).

Inhibition of Candida Attachment to the GI Tract and Systemic Spread By Saccharides In vivo:

The passage of viable C.albicans through the GI mucosa into the bloodstream is considered to be an important mechanism leading to systemic candidosis (246-248). Association of the yeast with intestinal mucosa is believed to be the very first stage in the colonisation and subsequent dissemination from the GI tract (180,198,200,212). Consequently, an understanding of the mechanism of interaction between the yeast and the GI mucosa could shed some light on how the organisms colonise the GI tract and act as the primary setting for disseminated candidosis (17). Furthermore, investigation related to blockage of yeast adherence to the GI mucosa are of obvious clinical connotations.

Work on the inhibition of candidal adherence to murine vaginal mucosa in vivo was initiated by Segal and co-workers (278,330). These workers demonstrated that treatment of the vaginas of mice before yeast inoculation with either the Candida cell wall component chitin; or its constituent N-acetylglucosamine (GlcNAc); or its derivative, (designated as chitin soluble extract [CSE]), blocked the attachment of C.albicans to the vaginal mucosal surfaces and thereby prevented vaginal infection (278). Treatment of mice with CSE or
GLcNAc after inoculation of the yeast did not prevent infection. These authors used normal cycling mice rather than estrogen-treated animals. One drawback of this animal model is that the induced infection is short lived and unstable. Consequently, the effect of the inhibitors used could not be fully appreciated. To avoid this shortcoming, subsequent studies used estradiol-treated mice in which a persistent candidal vaginitis was induced (330). The results obtained confirmed earlier findings in that CSE was found to be an effective measure for preventing candidal vaginitis (330). Chemical analysis of CSE and its two fractions [FI and FII] showed that CSE contain over 70% of proteins, concentrated mostly in FII (279). In addition, 3% of amino-sugars were detected both in the whole CSE and in FI, and lipids in the unfractioned CSE and both fractions (279).

In this study, infant mice model [5 to 6 days old] (251) were used to establish GI and systemic candidosis and explored the effect of Candida cell wall component on the GI transient colonisation and dissemination and as a possible means for prevention of infection. Infant mice were used in this study rather than adults because oral-intragastric inoculation of infant mice with C.albicans leads to GI and systemic candidosis (225,226,251,293). Adult mice are resistant to colonisation unless they are pre-treated with antibiotics (247). Heavy colonisation of the GI tract and visceral organs of infant mice follows oral inoculation with C.albicans (225,226,293). The GI infection persists in survivors to an age at which the animals are normally resistant. Mice with persistent infections, therefore, were used to study the effects of saccharide treatment on the colonisation and dissemination of C.albicans.

Our in vivo studies provide evidence that treatment of infant mice with mannose, GLcNAc or CSE, which are components of the Candida cell wall (106,140,153,178), leads to a remarkable reduction in systemic spread of C.albicans from the gut to visceral organs, particularly to the kidneys and spleen. This was true for the number of positive body organs infected, and for levels of colonisation. In addition, the levels of yeast colonisation of the stomach and intestine were generally
lower than those obtained for the control [ the levels of stomach colonisation by *C. albicans* in mannose and GLcNAc -treated animals were lower and the counts were 2.5-fold to 22-fold lower than the control values]. This inhibition was most significant when the treatment was carried out two days prior to yeast inoculation with a daily saccharide dose throughout the study period. Although a single dose of saccharides administered 30 min prior to yeast inoculation did reduce systemic spread of *Candida* to viceral organs, such a dosing regimen increased the transient colonisation of the stomach and intestine. When mice were treated post-inoculation, no inhibitory effect was observed. These results indicate that the dosing regimen employed for saccharide treatment is important. Treatment for long period to yeast inoculation is preferable to a single dose treatment. In addition, post-*Candida* inoculation treatment will not prevent colonisation and dissemination.

The mechanism/s of how saccharide treatment interfered with the GI colonisation and dissemination of *Candida* is not clear. However, these substances could bind to the GI epithelial mucosa and thereby, block the attachment of *C. albicans*, preventing or reducing the level of GI colonisation and subsequent systemic spread. Another possibility is the immune response of the GI mucosa to saccharides. It is known that chitin and its derivatives (331), GLcNAc (332), and mannose (333) exert immunopotentiating effects on animals challenged with tumor cells or with pathogenic microbes. Also, Suzuki *et al*. (331) have shown that both chitin and chitosan are able to exhibit immunopotentiating action for lethal challenge of *C. albicans* thus exhibiting a protective effect on mice administered with these polysaccharides. Possibly saccharides stimulate the mucosal immune response triggering protective immunological reactions which reduce the level of colonisation and dissemination. Whether one or both of these possibilities, or any other mechanism/s, are involved in the protection process is yet to be elucidated. The complexity of this problem is only starting to emerge.
Kennedy et al. (261) suggested five distinct mechanisms for the association of \textit{C.albicans} with GI mucosal surfaces. These included: adhesion to epithelium, adhesion to mucus, co-adhesion to adherent fungi, co-adhesion to adherent bacteria, and entrapment in the mucous gel overlying the epithelium. In the same study, Kennedy et al. found that cell-surface hydrophobicity of \textit{C.albicans} not to play a role in \textit{Candida} adhesion to intestinal mucosa. The predominant association mechanisms appeared to be entrapment in the mucous gel, and adhesion to mucus and the epithelium (261). Cole et al. (334) showed that in the absence of an immunocompromising treatment, \textit{Candida} is primarily localised in the stomach and intestines of infant mice [6-day-old] at 20 days post-inoculation. Cultures of homogenates of the esophagus of most animals, and homogenates of the liver, lungs, spleen and kidneys of all animals, proved negative for \textit{C.albicans}. When mice were immunocompromised with cortisone acetate and cyclophosphamide, a high density of invasive hyphae was observed and cultures of the homogenised stomach showed a 100-fold increase in colony forming unit of \textit{C.albicans} compared with stomach homogenates of infected but non-immunocompromised controls (334). In addition, homogenates of esophagus and selected body organs of most immunocompromised mice examined were positive for \textit{C.albicans} (334). Cole et al. suggested that the infant mice model may be particularly useful both for exploring methods which may prevent dissemination of \textit{C.albicans} from localised foci of colonisation in the GI tract after exposure of the host to immunocompromising drugs, and for testing the efficacy of anti-\textit{Candida} drugs in clearance of the pathogen from body organs with established fungal abscesses (334).

Obviously \textit{Candida} adhesion is a complex biological phenomenon governed by a multiplicity of mechanisms which, depending upon the mucosal surface involved, are partially or wholly functional. A number of points remain to be investigated in order to elucidate clearly the molecular basis of the adhesion mechanism/s. The affinity and number of binding sites involved in \textit{Candida} adhesion to epithelial tissues is essential for a complete and accurate description of the phases of adhesion.
Identification of surface factors serving as Candida adhesins or mucosal receptors, the nature of binding which takes place between these components and the nature of the adhesive events between Candida, epithelial tissues, and other bridging intermediates, is needed. In this respect screening (and/ or induction) of C. albicans isolates for adherence-defective strains is of prime importance for the elucidation of adherence mechanism/s and the contribution of this phenomenon to pathogenicity.

In conclusion, results obtained in this study suggest that antifungal drugs tested, at sub-inhibitory concentration, have multiple effects on Candida including reduced adhesion to epithelial cells, germination inhibition and stimulating the excretion of extracellular polymeric materials known to mediate adherence. The combined antimycotic and anti-adherence properties shown by octenidine and piritenidine, and the fact that they cause extensive leakage of cytoplasmic contents from the cells which was correlated with major morphological and ultrastructural changes in the yeast, point to the possibility of using these drugs in the control and prevention of infections due to these yeasts.

The in vivo results provide evidence that saccharides known to inhibit adherence of yeast in vitro interfere with the colonisation and dissemination of the GI tract by C. albicans in experimental animals. They also stress the fact that more in vivo studies are required to reveal the mechanism/s involved in order that blockage of adherence as an approach could be of further practical value in prophylaxis and treatment of candidosis.
References


57. Polak, A. and Scholer, H.J. (1973). Fungistatic activity, uptake and incorporation of 5-fluorocytosine in Candida albicans as influenced by pyrimidines and purines. II. Studies on distribution and incorporation,


human buccal epithelial cells. *Infect. Immun.*, 21, 64-68.


Figure 1. Growth curves of *C. tropicalis* KCCC 13605 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●—●); 1/4x MIC (○—○); 1/2x MIC ( ■—■ ) and 1x MIC (□—□) of AMB.

Figure 2. Growth curves of *C. tropicalis* KCCC 13605 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●—●); 1/4x MIC (○—○); 1/2x MIC ( ■—■ ) and 1x MIC (□—□) of NY.
Figure 1

Figure 2
Figure 3. Growth curves of *C. tropicalis* KCCC 13605 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●--●); 1/4 x MIC (O---O); 1/2 x MIC (■---■) and 1x MIC (□---□) of MN.

Figure 4. Growth curves of *C. tropicalis* KCCC 13605 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●--●); 1/4 x MIC (O---O); 1/2 x MIC (■---■) and 1x MIC (□---□) of 5-FC.
Figure 5. Growth curves of C. *Kefyr* KCCC 13709 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●—●); 1/4 x MIC (○—○); 1/2 x MIC (■—■) and 1x MIC (□—□) of AMB.

Figure 6. Growth curves of C. *Kefyr* KCCC 13709 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●—●); 1/4 x MIC (○—○); 1/2 x MIC (■—■) and 1x MIC (□—□) of NY.
Figure 7. Growth curves of C. Kefyr. KCCC 13709 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●--●); 1/4 x MIC (○--○); 1/2 x MIC ( ■--■ ) and 1x MIC (□--□) of MN.

Figure 8. Growth curves of C. Kefyr. KCCC 13709 grown at 37°C as a shake culture in YNB medium supplemented with 2.5% glucose (w/v). The arrow represent time at which drugs were added, 0.0 (●--●); 1/4 x MIC (○--○); 1/2 x MIC ( ■--■ ) and 1x MIC (□--□) of 5-FC.
Figure 7

Figure 8
APPENDIX II

STATISTICAL TREATMENT OF DATA
1. Mean:

The mean is the value obtained by adding all the measurements and dividing by the number of measurements. The formula for the mean is:

\[
\mu = \frac{\sum x}{n}
\]

where \( \mu \) (Greek letter "mu") stands for the mean
\( x \) stands for each of the individual observations
\( \sum \) (summation sign) indicates the operation of summing all values of \( x \)
\( n \) stands for the number of observations.

2. Standard Deviation (SD):

The basic formula for standard deviation is

\[
SD = \sqrt{\frac{\sum(x - \mu)^2}{n}}
\]

where \( x \) stands for each of individual observations
\( \sum \) indicates the operation of summing all values of \( x \)
\( \mu \) stands for the mean of all measurements
\( n \) stands for the number of observations.

Find the square root of \( \frac{(x - \mu)^2}{n} \).

3. Standard Error of Difference Between Means (SE)

\[
SE\ \text{diff} = \sqrt{\frac{SD_1^2 + SD_2^2}{n_1 \cdot n_2}}
\]

Square the standard deviation of sample 1 (SD1) and divide by the number of observations in the sample (n1),
square the standard deviation of sample 2 (SD2) and divide by the number of observations in the sample (n2), then take the square root. This will give the SE difference between the two means.

4. Student's t test:

The t distribution is mainly used for testing hypotheses and finding confidence intervals for means, given small samples from normal distributions. Its foundations were laid by W.S. Gosset under the pseudonym "student" (1908) so that it is sometimes known as student's t test. To evaluate the differences in the adherence values, the number of yeast cells adhering to every BEC was counted for 100 BEC taken at random. Then Student's t test was used by SPSS computer batch system on the means of those 100 observations to evaluate the differences in the adherence values (a sample of statistical treatment of data is enclosed).
SPSS DACH SYSTEM

SPSS FOR SPERRY UNIVAC 1100 EXEC 7, VERSION 4, RELEASE 9.0-W2.0, FEBRUARY 1983

SPACE ALLOCATION** ALLOWS FOR.. 37 TRANSFORMATIONS
WORKSPACE 7375 WORDS 150 RECODE VALUES + LAG VARIABLES
TRANSPACE 1125 WORDS 300 IF/COMPUTE OPERATIONS

1. RUN NAME SPSS RUN T-TEST
2. FILE NAME KSTD11A*KMA11G2
3. VARIABLE LIST SLNO,EXPNO,SUBGR,CC,HP,OP
4. INPUT MEDIUM DISK
5. INPUT FORMAT FIXED(F3.0,F2.0,F2.0,F2.0)

ACCORDING TO YOUR INPUT FORMAT, VARIABLES ARE TO BE READ AS FOLLOWS

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THE INPUT FORMAT PROVIDES FOR 6 VARIABLES. 6 WILL BE READ
IT PROVIDES FOR 1 RECORDS ("CARDS") PER CASE. A MAXIMUM OF 13 "COLUMNS" ARE USED ON A RECORD.

5. Y OF CASES UNKNOWN
7. *SELECT IF (SUBGR EQ 1)
3. T-TEST PAIRS=CC,HP/CC,OP,HP,OP

***** T-TEST PROBLEM REQUIRES 42 WORDS OF WORKSPACE *****

7. READ INPUT DATA

AFTER READING 200 CASES FROM SUBFILE KSTD11A, END OF DATA WAS ENCOUNTERED ON LOGICAL UNIT #8
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**FILE** KST01A (CREATION DATE = 10/16/83) *KHAlIDZ

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CC=CONTROL

QP= 1/4 MIC Pirtenidine

HP= 1/2 MIC Pirtenidine


Effects of Sub-Inhibitory Concentrations of Antifungal Agents on Adherence of Candida spp. to Buccal Epithelial Cells in Vitro

Die Wirkung subinhibitorischer Antimykotika-Konzentrationen auf die Adhärenz von Candida-Arten an Epithelzellen der Mundschleimhaut

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Key words: Candida — antifungal agents — epithelial cells — adherence

Schlüsselwörter: Candida — Antimykotika — Epithelzellen — Adhärenz

Summary: The adherence of three Candida spp. to human buccal epithelial cells following treatment of the yeast with subinhibitory concentrations of amphotericin B, nystatin, miconazole nitrate and 5-fluorocytosine was investigated in vitro. Preincubation of C. albicans, C. tropicalis or C. kefyr with these antifungals inhibited their adherence to varying degrees (reduction between 17% and 78% of the control value). Pretreatment of yeast for a short period (1 h) had less effect on adhesion than pretreatment for a long period (24 h). Furthermore, treating C. albicans with a combination of amphotericin B plus 5-fluorocytosine, both at 1/8 MIC level, led to stronger adherence inhibition than that obtained for yeast pretreated with either one alone at 1/4 MIC levels. Exposure of C. albicans to antifungals affected the outer cell envelope, as observed by scanning electron microscopy, it also suppressed germination significantly, again to a different extent depending on the antifungal used. Compared with the control grown yeasts, an increase in the excretion of extracellular polymer into the supernatant of yeast cultured with various antifungals was observed. Chemical composition of this material showed that it is mannoprotein in nature containing hexoses (85-90%) and protein (7-9%). Mannose was the major sugar making about 87% of the total carbohydrates. Our results suggest that antifungals, at sub-inhibitory concentrations, have multiple effects on Candida and point to the possibility of using these drugs in the prophylaxis against candidosis.

Zusammenfassung: Es wurde die Adhärenz von drei Candida-Arten an Epithelzellen der Mundschleimhaut nach Behandlung der Hefen mit subinhibitorischen Konzentrationen von Amphotericin B, Nystatin, Miconazolnitrat und 5-Fluorocytosin in vitro untersucht. Die Vorinkubation von C. albicans, C. tropicalis und C. kefyr mit diesen Antimykotika hemmte ihre Adhärenz in unterschiedlichen Graden (zwischen 17 und 78% Reduktion der Kontrollwerte). Die nur kurzzeitige Vorbehandlung von 1 h...
had a smaller effect on adherence than the 24 h-preparation. For this reason, the treatment method using *C. albicans* with the combination of Amphotericin B and 5-FC, both in 1/8 of the MHK, resulted in a stronger adherence-inhibition compared to the treatment method with a combination of different antifungals in 1/4 of the MHK. The exposure of *C. albicans* to different antifungal agents affected the adherence of yeast and contradictory. Sobel et al. (34) showed that pre-incubation of this yeast with ketoconazole at sub-inhibitory levels did not affect the adherence of yeast or germ-tube formation. In contrast, Macura (20) showed this azole to be effective both at the therapeutic and sub-inhibitory concentrations. Similar contradictory results were reported for the polyenes. In one study, pre-incubation of *C. albicans* with nystatin, at MIC and sub-MIC levels, did not have a significant effect on adherence (4). While in another study, nystatin pre-treatment, at both therapeutic and sub-inhibitory levels, inhibited yeast adhesion considerably (20). Likewise, different workers reported contrasting effects for 5-fluorocytosine on the adherence of *Candida* (4, 20). This prompted us to carry out an investigation into the effect of amphotericin B, nystatin, miconazole nitrate and 5-fluorocytosine on the adherence of various *Candida* species to buccal epithelial cells in vitro.

**Materials and Methods**

**Fungus**

Three species of *Candida* were used: *C. albicans*, *C. tropicalis* and *C. kefyr*. Two strains of the first species were investigated: *C. albicans* ATCC 10231, was obtained from the American Type Culture Collection, Rockville, Md., as a lyophilized culture. The other strain of *C. albicans* KCCC 14172, as well as *C. tropicalis* KCCC 13605 and *C. kefyr* KCCC 13709 were isolated on Sabouraud dextrose agar medium (Difco laboratories, Detroit, Mich) from the oral cavities of patients under-going head and neck radiation therapy at the Kuwait Cancer Control Centre (13). The identities of these isolates were confirmed as described earlier (6, 14)
Antifungals

Antifungal agents used were: Amphotericin B (AMB), nystatin (NY), miconazole nitrate (MN) and 5-fluorocytosine (5-FC). All drugs were purchased from Sigma chemical company (Saint Louis, Missouri).

Determination of minimum inhibitory concentration (MIC)

An agar dilution method was used to determine the MIC for various Candida spp. used. Serial dilutions were prepared for the antifungal agents in molten Sabouraud dextrose agar (for 5-FC in yeast nitrogen base Difco), then poured and left to solidify. Plates were inoculated (10^7 cells/ml) with overnight cultures of the organisms using a multipoint inoculator (Denley). Plates were incubated at 37°C for 48 h, when the MIC values were recorded. The MIC was defined as the first dilution showing no visible growth.

Effect of antifungal agents on growth

Each of four antifungal agents was tested for growth inhibition of C. albicans KCCC 14172 and all drugs were added at 1/4 MIC levels. Growth rates were measured in the presence and absence of test drugs. C. albicans was inoculated into 25 ml of Sabouraud dextrose broth and incubated overnight in a rotary shaker at 37°C. The cells were then centrifuged and resuspended in a small volume of fresh medium. This suspension was used to inoculate 5 x 50 ml flasks of fresh medium. Drugs were introduced into the media 2 h after inoculation as cells entered log phase. The cultures were incubated on an orbital shaker at 37°C. Aliquots were removed at intervals, and the growth was determined spectrophotometrically at 420 nm (SP6-500 Pye Unicam). Since all antifungal agents used were dissolved in a minimal amount of dimethylsulfoxide (DMSO), an equivalent amount of DMSO was added to control flasks as well as to a blank flask which contained media only.

Scanning electron microscopy (SEM)

C. albicans KCCC 14172 was grown in flasks containing 100 ml yeast nitrogen base (Difco) supplemented with 2.5% glucose (w/v). Control and test flasks, each containing 1/2 or 1/4 MIC level, were grown in a shake culture at 37°C and for 24 h. Cells were then prepared for SEM and coated with gold-palladium alloy (11). Samples were examined in a stereoscopic electron microscope (Novascan 30) at an angle of -5°C.

Germ-tube formation

C. albicans ATCC 10231 as well as C. albicans KCCC 14172 cells grown in the absence and presence of 1/4 MIC levels of various antifungal agents were washed with phosphate buffered saline (PBS), pH 7.2, diluted into heat inactivated newborn calf serum (Gibco, Grand Island, N.Y) and incubated at 37°C. At zero, 60 and 120 min, samples were removed and added to an equal volume of 1% glutaraldehyde in PBS for fixation. The number of yeast with germ-tubes was determined microscopically (35).

Preparation of Candida cells for adherence studies

The effect of sub-inhibitory concentrations of various antifungal agents on the adherence of different Candida spp. to buccal epithelial cells (BEG) was studied by incubating the yeast in the absence and the presence of 1/4, 1/8 and 1/16 MIC level of each antifungal agent. The yeast was incubated in a rotary shaker with 160 rpm for 24 h at 37°C. Yeast cells were then harvested, washed twice with Hanks balanced salt solution (HBSS) (25), and standardized to 1 x 10^7 cells/ml. These cells were used in the adherence assay.

In other experiments antifungal agents were diluted into overnight cultures of yeast cells.
grown in yeast nitrogen base supplemented with 2.5% glucose (w/v) and adjusted to pH 6.0, to produce final concentration of 1/4, 1/8 and 1/16 MIC levels. These suspensions were then incubated in a rotary shaker at 37°C for additional 60 min. Subsequently, yeast cells were washed with HBSS and standardised ready for adherence assays. The effect of combination of AMB and 5-FC was monitored by growing *C. albicans* KCCC 14172 in yeast nitrogen base medium supplemented with 2.5% glucose (w/v) in an orbital shaker at 37°C for 24 h. Flasks containing 1/4 of the same medium were inoculated with 50 ml of the overnight culture. Control and test flasks, each containing 1/4 MIC levels of various antifungal agents or a combination of AMB and 5-FC (each at 1/8 MIC), were incubated for 24 h at 37°C. Following incubation, yeast cells were harvested by centrifugation, washed twice with sterile distilled water in pre-weighed centrifuge tubes. The tubes were then left to dry overnight at 75°C and the dry weight determined. EP was isolated from culture supernatants by precipitation with acetone and according to the method of McCourtie and Douglas (22).

Quantitative analysis of EP extracted was done by descending paper chromatography on Whatman No. 4 paper of acid hydrolysates in butan-1-ol/ethyl acetate/acetic acid/pyridine/water (30:30:5:25:15, by vol). Hydrolys products were identified by comparing their chromatographic behaviour with that of authentic standards of sugars (23). For quantitative analysis, protein was determined by the Lowry method, while total carbohydrate was estimated spectrophotometrically according to the procedures of Dubois et al. (9), using mannose as a standard.

**Results**

**Minimum inhibitory concentration**

The inhibitory activity of antifungal agents used against *C. albicans* showed that amphotericin B was the most active agent (MIC 0.5 μg/ml) followed by nystatin (MIC...
Figure 1: Inhibition of *C. albicans* KCCC 14172 growth in liquid cultures by various antifungal agents. All drugs were added at 1/4 of their MIC. The arrow represents time at which drugs were added: control •, nystatin Δ, amphotericin B ○, 5-fluoroconazole ▲, and miconazole □.

Figure 2: Effect of amphotericin B (at 1/4 MIC) on the morphology of *C. albicans* KCCC 14172. A: Control. B: Amphotericin B.
<table>
<thead>
<tr>
<th>Organisms</th>
<th>Control</th>
<th>1/4 MIC</th>
<th>1/8 MIC</th>
<th>1/16 MIC</th>
<th>Control</th>
<th>1/4 MIC</th>
<th>1/8 MIC</th>
<th>1/16 MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>468</td>
<td>286</td>
<td>&lt;0.001</td>
<td>328</td>
<td>&lt;0.001</td>
<td>410</td>
<td>N S</td>
<td>510</td>
</tr>
<tr>
<td>ATCC 10231</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C. albicans</td>
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<td>359</td>
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<td>387</td>
<td>&lt;0.001</td>
<td>440</td>
<td>N S</td>
<td>536</td>
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<tr>
<td>C. tropicalis</td>
<td>386</td>
<td>216</td>
<td>&lt;0.001</td>
<td>255</td>
<td>&lt;0.001</td>
<td>342</td>
<td>N S</td>
<td>360</td>
</tr>
<tr>
<td>C. kefyr</td>
<td>264</td>
<td>189</td>
<td>&lt;0.001</td>
<td>192</td>
<td>&lt;0.001</td>
<td>214</td>
<td>N S</td>
<td>295</td>
</tr>
</tbody>
</table>

| Table 1: The effect of pre-incubation of various Candida spp. in sub-inhibitory concentrations of amphotericin B on adherence to buccal epithelial cells |

Mean number of adherent yeasts per 100 epithelial cells

<table>
<thead>
<tr>
<th>a</th>
<th>P values compared with control</th>
</tr>
</thead>
<tbody>
<tr>
<td>N S</td>
<td>not significant</td>
</tr>
<tr>
<td>*</td>
<td>Values between brackets are ± SE</td>
</tr>
</tbody>
</table>

<table>
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<tr>
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<th>1/8 MIC</th>
<th>1/16 MIC</th>
<th>Control</th>
<th>1/4 MIC</th>
<th>1/8 MIC</th>
<th>1/16 MIC</th>
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<tbody>
<tr>
<td>C. albicans</td>
<td>510</td>
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<td>390</td>
<td>&lt;0.001</td>
<td>451</td>
<td>N S</td>
<td>505</td>
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<tr>
<td>C. albicans</td>
<td>563</td>
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<td>&lt;0.001</td>
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<td>&lt;0.001</td>
<td>416</td>
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<tr>
<td>C. tropicalis</td>
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<td>270</td>
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<tr>
<td>C. kefyr</td>
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<td>&lt;0.05</td>
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</table>

<table>
<thead>
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<th>a</th>
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</tr>
</thead>
<tbody>
<tr>
<td>N S</td>
<td>not significant</td>
</tr>
<tr>
<td>*</td>
<td>Values between brackets are ± SE</td>
</tr>
</tbody>
</table>
Table 3: The effect of pre-incubation of various *Candida* spp. in sub-inhibitory concentrations of miconazole nitrate on adherence to buccal epithelial cells

<table>
<thead>
<tr>
<th>Organisms</th>
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<th>1/16 MIC</th>
<th>Control</th>
<th>1/4 MIC</th>
<th>1/8 MIC</th>
<th>1/16 MIC</th>
<th>Control</th>
<th>1/4 MIC</th>
<th>1/8 MIC</th>
<th>1/16 MIC</th>
<th>Control</th>
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<td>235</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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<td></td>
<td>(22)</td>
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<td>(23)</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
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<td>&lt;0.001</td>
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<td>315</td>
<td>&lt;0.001</td>
<td>208</td>
<td>&lt;0.001</td>
<td>234</td>
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<td>(27)</td>
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<td>(17)</td>
<td></td>
<td>(15)</td>
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<td>(15)</td>
<td></td>
<td>(15)</td>
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<td>(15)</td>
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<tr>
<td><em>C. tropicalis</em></td>
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<td>276</td>
<td>&lt;0.001</td>
<td>294 N S</td>
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<td>201</td>
<td>&lt;0.001</td>
<td>228</td>
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<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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<tr>
<td><em>C. kefyr</em></td>
<td>263</td>
<td>169</td>
<td>&lt;0.001</td>
<td>201 N S</td>
<td>222</td>
<td>N S</td>
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<td>KCCC 13709</td>
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<td>(15)</td>
<td></td>
<td>(18)</td>
<td></td>
<td>(20)</td>
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<td></td>
<td>(23)</td>
<td></td>
<td>(20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\* P values compared with control
\* N S not significant
\* Values between brackets are + SE

Table 4: The effect of pre-incubation of various *Candida* spp. in sub-inhibitory concentrations of 5-fluorocytosine on adherence to buccal epithelial cells

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Control</th>
<th>1/4 MIC</th>
<th>1/8 MIC</th>
<th>1/16 MIC</th>
<th>Control</th>
<th>1/4 MIC</th>
<th>1/8 MIC</th>
<th>1/16 MIC</th>
<th>Control</th>
<th>1/4 MIC</th>
<th>1/8 MIC</th>
<th>1/16 MIC</th>
<th>Control</th>
<th>1/4 MIC</th>
<th>1/8 MIC</th>
<th>1/16 MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>447</td>
<td>269</td>
<td>&lt;0.001</td>
<td>343</td>
<td>&lt;0.001</td>
<td>362</td>
<td>&lt;0.001</td>
<td>285</td>
<td>&lt;0.001</td>
<td>312</td>
<td>&lt;0.001</td>
<td>360</td>
<td>&lt;0.001</td>
<td>360</td>
<td>&lt;0.001</td>
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<tr>
<td>ATCC 10231</td>
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<td>(17)</td>
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<td>(19)</td>
<td></td>
<td>(17)</td>
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<td>(13)</td>
<td></td>
<td>(14)</td>
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<td>(14)</td>
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<tr>
<td><em>C. albicans</em></td>
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<td>&lt;0.001</td>
<td>438 N S</td>
<td>524</td>
<td>284</td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
<td>320</td>
<td>&lt;0.001</td>
<td>320</td>
<td>&lt;0.001</td>
<td>320</td>
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<tr>
<td><em>C. tropicalis</em></td>
<td>371</td>
<td>262</td>
<td>&lt;0.001</td>
<td>286</td>
<td>&lt;0.05</td>
<td>345 N S</td>
<td>340</td>
<td>232</td>
<td>&lt;0.001</td>
<td>255</td>
<td>&lt;0.001</td>
<td>262</td>
<td>&lt;0.001</td>
<td>262</td>
<td>&lt;0.001</td>
<td>262</td>
</tr>
<tr>
<td>KCCC 13605</td>
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<td>(17)</td>
<td></td>
<td>(19)</td>
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<td>(14)</td>
<td></td>
<td>(14)</td>
<td></td>
<td>(14)</td>
</tr>
<tr>
<td><em>C. kefyr</em></td>
<td>300</td>
<td>230</td>
<td>&lt;0.05</td>
<td>274 N S</td>
<td>286</td>
<td>N S</td>
<td>285</td>
<td>189</td>
<td>&lt;0.001</td>
<td>214 N S</td>
<td>&lt;0.05</td>
<td>250 N S</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>KCCC 13709</td>
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<td></td>
<td>(17)</td>
<td></td>
<td>(12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\* P values compared with control
\* N S not significant
\* Values between brackets are + SE
agents led to a more drastic damage to the yeast with cell collapse, lysis and concurrent leakage of cytoplasmic material with eventual death.

**Influence on germ-tube induction**

The results shown in Figure 3 demonstrate that germ-tube formation in newborn calf serum was significantly suppressed when either of two strains of *C. albicans* was incubated for 24 h in the presence of 1/4 MIC levels of various antifungal agents. The pattern of germ-tube inhibition was NY > AMB > MN > 5-FC (Fig 3A and B).

**Effect on adherence**

Treatment of *C. albicans* (two strains), *C. tropicalis* and *C. kefyr* with four different antifungal agents affected the adherence of yeasts to buccal epithelial cells (Tables 1 to 4). In general, pre-treatment of *Candida* with antifungals for a short period (1 h) has less effect on adherence than pre-treatment for a long period (24 h). Comparison of adherence-inhibiting activity of antifungals used in this study gave the following pattern of activity, NY > MN > 5-FC > AMB (Table 5). This pattern holds for yeasts pre-treated with antifungals for a long period (24 h) and applied to the two *C. albicans* strains tested. However, a different pattern of activity emerged when yeast cells were pre-treated with antifungals for a short period (1 h) (Table 5) A difference also existed between the two strains tested with one-hour pre-treatment. Pre-treatment of *C. albicans* with a combination of AMB and 5-FC, both at 1/8 MIC, led to stronger adherence-inhibition than that obtained for yeast pre-treatment with either one alone at higher concentrations (1/4 MIC) (Table 6). Exposure to the combination for a short period resulted in an almost additive effect, while treatment for a long period led to a considerable reduction in adherence of *Candida*, as compared to exposure to each drug alone, but less than additive (Table 6).
Table 5: Percent reduction of adherence of Candida spp to buccal epithelial cells following pre-incubation in 1/4 MIC of antifungal agents

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>Pre-incubation time</th>
<th>C. albicans ATCC 10231</th>
<th>C. albicans KCCC 14172</th>
<th>C. tropicalis KCCC 13605</th>
<th>C. kefyr KCCC 13709</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>24 h</td>
<td>24 h</td>
<td>24 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Amphotericin B</td>
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<td>26</td>
<td>44</td>
<td>50</td>
<td>28</td>
</tr>
<tr>
<td>Nystatin</td>
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<td>44</td>
<td>42</td>
<td>41</td>
</tr>
<tr>
<td>Miconazole nitrate</td>
<td>45</td>
<td>50</td>
<td>34</td>
<td>36</td>
<td>41</td>
</tr>
<tr>
<td>5-Fluorocytosine</td>
<td>40</td>
<td>44</td>
<td>29</td>
<td>23</td>
<td>34</td>
</tr>
</tbody>
</table>

Table 6: Effect of pre-incubation of C. albicans KCCC 14172 in a combination of amphotericin B plus 5-fluorocytosine on adherence to buccal epithelial cells

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>1 h Pre-incubation</th>
<th>24 h Pre-incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast adhering to 100 BEC (mean ± SE)</td>
<td>E&lt;sup&gt;a&lt;/sup&gt; % reduction</td>
<td>Yeast adhering to 100 BEC (mean ± SE)</td>
</tr>
<tr>
<td>Control</td>
<td>509 ± 22</td>
<td>532 ± 20</td>
</tr>
<tr>
<td>Amphotericin B (1/4 MIC)</td>
<td>407 ± 16 &lt;0.001 20</td>
<td>324 ± 15 &lt;0.001 39</td>
</tr>
<tr>
<td>5-Fluorocytosine (1/4 MIC)</td>
<td>352 ± 16 &lt;0.001 31</td>
<td>318 ± 16 &lt;0.001 40</td>
</tr>
<tr>
<td>Amphotericin B plus 5-Fluorocytosine (both at 1/8 MIC)</td>
<td>265 ± 15 &lt;0.001 48</td>
<td>251 ± 16 &lt;0.001 53</td>
</tr>
</tbody>
</table>

<sup>a</sup> Compared with control

Effect on the release of EP material

Table 7 shows that exposure of C. albicans KCCC 14172 to subinhibitory concentration of antifungals led to an increase in the excretion of EP material from this yeast. These polymers were extracted according to the method of McCourtie & Douglas (22). Similar to the results of these authors, chemical analysis of the polymer obtained in our study showed that they are mannoprotein in nature containing hexoses (85-90%) and protein (7-9%). Mannose was the major sugar, making about 85% of the total carbohydrate. The loss of EP were mostly under the influence of AMB plus 5-FC combination followed by 5-FC > MN > AMB > NY (Table 7).

For the same weight, the percentages of hexoses extracted from the supernatant of yeasts cultured with various antifungal agents were increased when compared to the control. In contrast, the percentages of protein present in the EP materials extracted were decreased.

The only exception to this is the composition of EP from supernatants of yeasts grown in the presence of a combination of AMB plus 5-FC, where the opposite was observed.

— 559 —
Sub-inhibitory levels of antifungal agents tested in this study significantly blocked, to varying degrees, the adherence of Candida spp. to BEC, depending on the antifungal used and pre-treatment time. Moreover, all the antifungals tested affected the outer cell surfaces and germination ability of C. albicans to different extents also. Although NY showed the highest inhibition activity for both adherence and germination, the two patterns do not exactly match. Furthermore, exposure to sub-inhibitory concentrations of various antifungals led to an accumulation of EP materials in the culture supernatant. The extent of accumulation was again drug-dependent. Interestingly, the most active antifungal and pattern of increased excretion in response to drug treatment was different from those obtained for both adherence and germination.

Differences observed in the activity vs. adherence, morphology, germination and excretion of EP materials as a response to pre-treatment with different antifungals are not unexpected, since the antifungals that have been studied, do not share the same mode of action. The antifungal activity of polyenes is related to their binding to sterols, particularly ergosterol. This interaction results in the formation of pores in the fungal membrane altering its permeability and leading to loss of cytoplasmic material and eventual death. By binding with yeast membrane sterols, polyenes lead to a decrease in hydrophobicity of the fungi which probably contributes to the observed reduction in adherence following treatment with these drugs. A plausible explanation of the discrepancy of results between AMB and NY, even though they are structurally similar, could be the heterogeneity of the mechanisms of polyene action.

Azole antifungals are characterized by heterogeneity of action mechanisms. However, the mode of action shared by all azoles involves the inhibition of ergosterol
fungal cell by the enzyme cytosine deaminase to 5-fluorouracil, which in turn, is phosphorylated to 5-fluorouridine monophosphate. The latter is phosphorylated further and incorporated into the yeast RNA with eventual disruption of protein synthesis (26, 28, 29). Inhibition of DNA synthesis is brought about by the formation of 5-fluorocytosine monophosphate. The latter is phosphorylated and incorporated into the yeast RNA with eventual disruption of protein synthesis (26, 28, 29).

The presence of multiple mechanisms that may act simultaneously in the process of adherence of Candida albicans epithelial cells may provide yet another reason for the differences observed between antifungal agents (16, 18). As shown in this investigation these agents affect, to varying degrees, various factors, which are known to influence the adherence process either directly (such as EP and other cell envelopes) (22, 38) or indirectly (such as germination) (17). Thus, it is quite difficult to compare the effect of various antifungal agents on the adherence of Candida per se without considering these points.

In conclusion, our results suggest that antifungal agents, tested at sub-inhibitory concentrations, have multiple effects on Candida albicans including reduced adhesion to epithelial cells, germination inhibition and stimulating the excretion of EP materials known to mediate adherence.

Acknowledgements: This project was partially supported by Grant No. SO038, Research Management Unit, Kuwait University. The help of Miss. R. Yassin in literature search is appreciated.

References


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Antimycotic effects of octenidine and pirtenidine

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*Department of Botany and Microbiology, Kuwait University, Kuwait; *Department of Biology, Maynooth College, Maynooth, Ireland

The effects of octenidine and pirtenidine on yeasts (in particular Candida albicans) have been studied. MIC and MCC values have been established as well as the inhibitory effects on growth, budding and germ tube formation. The drugs were shown to cause extensive leakage of cytoplasmic contents from the cells which was correlated with morphological and ultrastructural changes in the yeast.

Introduction

The increasing significance of fungal infections in man and other animals has been widely reported (Armstrong et al., 1975; Kiehn, Edwards & Armstrong, 1980; Odds, 1988). There are a number of factors which have contributed to this increased significance in recent years (Ghannoum, 1988; Odds, 1988). These include the widespread use of antibacterial antibiotics, the development and use of immunosuppressive agents in the treatment of organ transplant patients and patients suffering from autoimmune or immune deficiency diseases (Hurley, de Louvois & Mulhall, 1987). The dimorphic yeast Candida albicans is without doubt the most important pathogenic fungus to have increased in significance owing to these factors (Mirsy & Cutner, 1972).

The problems caused by this increase in fungal pathogenesis are of greater importance because of the relative lack of antifungal agents which are both effective and free of side effects (Speller, 1980). It is consequently very important to investigate thoroughly any new drug with antifungal potential, to establish its mode of action and clinical possibilities.

In this paper we describe some studies related to the antimycotic effects of two drugs, octenidine and pirtenidine (Figure 1) with some structural similarity to chlorhexidine, which have been developed for potential use as antibacterial mouthwashes. Although these were originally selected for their antibacterial properties, preliminary studies (Sedlock & Bailey, 1985; Whittaker & Ellabib, unpublished) established that they also exhibited antimycotic properties.

Materials and methods

Drugs

Octenidine hydrochloride [N,N’-(1 10 decanediyldi-[4H]-pyridinyl-4-ylidene)bis-(1-octanamine) dihydrochloride] and pirtenidine [N-(1-octyl-[4H]-pyridinylidene) octa-
Pirtenidine

Octenidine

Chlorhexidine

Figure 1. Structures of pirtenidine, octenidine and chlorhexidine

namine monohydrochloride} (Figure 1) are new drugs developed by Sterling-Winthrop Research Institute as antiseptic or antifungal agents. These drugs were gifts from Sterling-Winthrop.

Organisms

Yeast species used were: C. albicans KCCC 14172, C. tropicalis KCCC 13622 and C. pseudotropicalis KCCC 13709, which were isolated from the oral cavities of patients undergoing head and neck radiation therapy at Kuwait Cancer Control Centre, and lyophilized. The isolation and identification techniques used have already been described (Ghannoum et al., 1985a, b). C. albicans ATCC 10231 which was isolated from a patient suffering from bronchomycosis and obtained as a lyophilized sample from the American Type Culture Collection, USA and Saccharomyces cerevisiae NCYC 975 were also used. All the organisms were maintained on slopes of Sabouraud modified agar (Difco), stored at 4°C, and subcultured routinely.

Determination of minimum inhibitory concentrations (MIC) and minimum cidal concentrations (MCC)

Serial two-fold dilutions of the drugs ranging from 800 mg/l to 0.2 mg/l were prepared in tubes containing 10 ml of yeast nitrogen base (Difco) supplemented with 2-5%
Antimycotic effects of octenidine and pirenidine

dextrose (YNBD). These were inoculated with 0.01 ml of a fresh overnight broth culture (approx. 10^7 cfu/ml) and then incubated at 37°C for 24 h. The MIC was noted as the lowest concentration showing no visible turbidity. One loopful (approx. 0.01 ml) of the broth tubes showing no visible growth was further subcultured on to a Sabouraud agar plate to determine the MCC. The plates were incubated at 37°C for 24 h and then checked for growth. The MCC was recorded as the lowest concentration yielding no colonies.

Effects on growth

Inocula of *C. albicans* KCCC 14172 and *S. cerevisiae* were grown overnight at 37°C with rotary agitation in YNBD. The cells were centrifuged and resuspended in a small volume of fresh medium. This suspension was used to inoculate (10^4 cells/ml) 4 × 100 ml of fresh medium containing O (control), ½ × MIC, ¼ × MIC and 1 × MIC of the drugs. The flasks were incubated at 37°C. Samples were withdrawn at 60-min intervals, and the rate of growth of shaken cultures was followed by determining the optical density at 420 nm (SP6-550 Pye Unicam).

Action on budding cells

*C. albicans* KCCC 14172 and *S. cerevisiae* were maintained on YNBD for 48 h. The cells were centrifuged and washed three times with distilled water. Yeast cells (10^7/ml) were inoculated into flasks containing 20 ml of Eagle’s medium (which supports bud formation of yeasts) with and without drugs. Flasks were incubated at 25°C on a gyratory shaker at 200 rpm. Samples were taken at intervals and scored for bud formation by counting the mean number of yeasts forming buds in every 300 yeast cells.

Effects on germ tube formation

*C. albicans* KCCC 14172 cells grown with and without drugs were washed three times with distilled water. An inoculum from each preparation was added to tubes containing calf serum (Gibco) and incubated in a shaking water bath at 37°C. Samples were taken at intervals and added to an equal volume of 1% glutaraldehyde in phosphate buffer-saline (PBS) for fixation. The number of yeasts with germ tubes was determined microscopically (Soll, Bedell & Brummel, 1981).

Leakage of intracellular material

Equal volumes (5 ml) of octenidine and pirenidine solutions (O (control), ¼ × MIC, ½ × MIC and 1 × MIC) and cell suspensions were mixed to give a final cell concentration of 1 mg (wet weight)/ml and incubated at 24°C. At intervals cells were removed by centrifugation (7000 g, 5 min). Cellular exudates were determined by direct spectrophotometric measurement of the material absorbing at 260 nm in the supernatant.

Scanning electron microscopy (SEM)

*C. albicans* KCCC 14172 and *S. cerevisiae* were grown in flasks containing 100 ml YNBD with and without the drugs in shake cultures at 37°C for 1, 3, 6 and 24 h. Cells were then prepared for SEM using the technique of Ghannoum & Al-Khars (1984).
Transmission electron microscopy (TEM)

Cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.0, at 4°C for 2 h. Cells were placed in freshly made 2% (w/v) KMnO₄ solution at 4°C for 2 h. The cells were centrifuged at 4000 rpm for 5 min and placed in a fresh solution of KMnO₄ for 2 h. They were washed five times with distilled water. The cells were then placed in a solution containing 1% (w/v) potassium dichromate and 1% (w/v) uranyl acetate for 2 h at 4°C. These were washed several times with distilled water and embedded in agar, left to set and cut into small cubes (0.5-1 mm³) which were dehydrated through an ethanol series. The 100% ethanol was replaced with propylene oxide twice for 20 min and the sample was embedded in Epon by graded impregnation. Sections were obtained using an ultramicrotome, counterstained with lead citrate, and observed under a JEOL 100 CX microscope.

Results

Inhibitory and mycocidal effects

The MICs of octenidine and pirtenidine for each yeast strain tested were between 1.5 and 3.0 mg/l. There was more variation in the mycocidal effects of the drugs—S. cerevisiae being the most sensitive, with the MCCs being the same as the MICs. For the Candida strains MCCs were between 12.5 and 25 mg/l.

The effects of different concentrations of the drugs on the growth curves of C. albicans KCCC 14172 and S. cerevisiae showed that in all cases growth inhibition was concentration-dependent. Figure 2 shows the growth curves obtained with pirtenidine.
Antimycotic effects of octendine and pirtendine

Figure 3. Leakage of cytoplasmic material from *C. albicans* KCCC 14172 and *S. cerevisiae* induced by octendine. Octendine concentration (mg/l): *C. albicans* ▼, O (control), ●, 0.75 ( = 1 x MIC), ■, 1.5 ( = 1 x MIC), △, 2.0 ( = 2 x MIC), *S. cerevisiae* ▲, O (control), ○, 0.75 ( = 1 x MIC), □, 1.5 ( = 1 x MIC), △, 3.0 ( = 2 x MIC)

and *C. albicans*, with exponential growth in the presence of the drug but increased doubling times. Qualitatively similar results were obtained with both drugs and with both yeast species.

Both octendine and pirtendine were shown to inhibit the extensive budding initiated on transfer of either *C. albicans* KCCC 14172 or *S. cerevisiae* to Eagle's medium. The drugs also blocked germ tube formation in *C. albicans* on incubation in calf serum.

*Leakage of intracellular material*

Leakage of cytoplasmic material from *C. albicans* and *S. cerevisiae* cells inoculated into various octendine concentrations is shown in Figure 3. The drug induced a rapid
Figure 4. SEM of *C. albicans* KCCC 14172 treated with octenidine (1 × MIC). (a) Control, (b) grown 1 h in octenidine; (c) grown 24 h in octenidine.
leakage of cellular contents from the yeasts *S. cerevisiae* was more susceptible than *C. albicans*. Qualitatively similar results were observed with pirtenidine, although less UV-absorbing material was released.

**Electron microscopy**

The effects of growth of yeast in the presence of octenidine and pirtenidine have been studied by scanning and transmission electron microscopy. Figure 4 shows the effects of growth of *C. albicans* KCCC 14172 for 1 and 24 h in the presence of the minimal inhibitory concentration (1.5 mg/l) of octenidine. Control cells (Figure 4(a)) exhibited a smooth-walled appearance and were spherical to ovoid in shape. They were in the yeast form and showed clear evidence of budding. After 1 h of incubation (Figure 4(b)) some of the cells showed evidence of weakening in the cell envelope with indications of cell collapse in some cases. These effects increased in 3- and 6-h samples (not shown) which culminated after 24 h (Figure 4(c)) in extensive cell damage, lysis and collapse of cell structure and extrusion of cellular contents. Qualitatively similar results were observed using either drug and also with *S. cerevisiae*.

Transmission electron micrographs of control *C. albicans* cells (Figure 5(a)) showed typical budding cells with multi-layered cell wall and mitochondrial and nuclear profiles. Incubation of cells in the presence of 1.5 mg/l octenidine (Figure 5(b)) resulted in gross ultrastructure alterations. Cells tended to adopt irregular shapes and the cell walls became irregular in structure with deformities in the layering structure and apparent loss of internal cohesion. Cell membranes lost their integrity and cytoplasmic contents coagulated, giving rise to electron-dense and electron-thin regions. Similar observations were made when octenidine was replaced by pirtenidine.

**Discussion**

The results presented here clearly demonstrate that the drugs octenidine and pirtenidine exhibit potent mycostatic and mycocidal activities against a number of yeast strains including strains of the important pathogens *C. albicans* and *C. tropicalis*. They also show that the drugs have significant effects on cell growth and integrity as seen in electron microscopy and cell leakage studies. In most respects octenidine is quantitatively more potent than pirtenidine. The greater mycocidal sensitivity of *S. cerevisiae* relative to *C. albicans* could be directly related to drug-induced leakage of cytoplasmic contents. The effects observed parallel those reported for chlorhexidine (Bobichon & Bouchet, 1987). These workers speculated on the mechanism of action for chlorhexidine and concluded that coagulation of nucleoplasm was a critical event in the effect of this drug. No biochemical evidence was presented for this, however. In the cases of octenidine and pirtenidine we cannot as yet be certain of their primary target but gross changes in the cell envelope and leakage of cell contents begin at an early stage which suggests the possibility that the primary site of attack of the drugs is at the level of the cell wall or plasma membrane.

Inhibition of germ tube formation by the drugs is of interest as germ tube formation has been implicated in the adherence and pathogenicity of *C. albicans* (Kimura & Pearsall, 1980; Sobel, Muller & Buckley, 1984) which highlights their potential for clinical control of yeast infections.
Figure 5. TEM of C. albicans KCCC 14172 treated with octenidine (1 × MIC). (a) Control, (b) grown 24 h in octenidine.
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EFFECTS OF OCTENIDINE AND PIRTENIDINE ON ADHESION OF CANDIDA SPECIES TO HUMAN BUCCAL EPITHELIAL CELLS IN VITRO

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Summary—Adherence of Candida spp to buccal epithelial cells in vitro was significantly reduced after both short- and long-term periods of yeast exposure to sub-inhibitory concentrations of octenidine and pirtendine. In addition, the pretreatment of either Candida or the epithelial cells or both with the drugs reduced adherence, this being greatest when both types of cells were pretreated. No difference in adherence to buccal epithelial cells was observed between yeast from stationary or exponential phases and the drugs were effective in reducing the adherence of cells from either growth phase. The drugs also inhibited germ-tube formation, which might contribute to their effects on adherence as far as C. albicans is concerned.

Key words: Candida spp., antifungal agents, adherence, mouth.

INTRODUCTION

Oral candidosis now ranks among the most common of all fungal diseases affecting humans (Douglas, 1987) and investigation of any drug with potential for control or prevention of this disease is important. The drugs octenidine and pirtendine (Fig. 1) have been developed as potential antimicrobial/antiplaque agents for use in mouthwash formulations. These drugs are both alkyl-pyridylidyne-octanamine derivatives, which are broad-spectrum antibacterial agents and which have also been shown to be active against pathogenic yeasts and fungi (Sedlock and Bailey, 1985; Ghanmoum et al., 1989).

Adherence of Candida to epithelial cells is considered to be the initial stage in the infection (Douglas, 1987) and antifungal drugs that also interfere with this process could be of enhanced clinical value. Chlorhexidine, also used as an antibacterial mouthwash, has been shown to inhibit adhesion of Candida to buccal epithelial cells (Tobgi, Samaranyake and MacFarlane, 1987) and to denture-acrylic strips (McCourtie, MacFarlane and Samaranyake, 1985, 1986). We now describe a study on the effects of the drugs on adherence of Candida albicans, C. tropicalis and C. pseudotropicalis to human buccal epithelial cells.

MATERIALS AND METHODS

Organisms

Yeast species used were C. albicans ATCC 10231; C. albicans KCCC 14172, C. tropicalis KCCC 13622; C. pseudotropicalis KCCC 13709. The three last mentioned isolates were obtained from the mouths of patients undergoing head and neck radiation therapy at the KCCC. Identification, as described by Ghannoum et al. (1985), was carried out using an API 20C (API Laboratory Products Ltd, Basingstoke, England) kit and observation of germ-tube formation.

Media and chemicals

Growth of yeast was at 37°C in yeast nitrogen base broth (Difco, East Molesey, England) supplemented with 2.5% glucose, as described by Ghannoum and Abu Elleen (1987). Addition of drugs was as described in Results. Octenidine and pirtendine were provided by Sterling Winthrop Research and Development, Guildford, England. MIC values, which had previously been determined using the broth dilution technique of Shadomy and Espinel-Ingroff (1980), were as follows: for octenidine—KCCC 14172, 1.5 μg/ml; ATCC 10231, 3.0 μg/ml; KCCC 13622, 3.0 μg/ml; KCCC 13709, 1.5 μg/ml and for pirtendine—KCCC 14172, 3.0 μg/ml; ATCC 10231, 1.5 μg/ml; KCCC 13622, 3.0 μg/ml; KCCC 13709, 1.5 μg/ml.

Preparation of buccal epithelial cells

Buccal epithelial cells were collected from 6 healthy human volunteers, as described by Ghannoum and Abu Elleen (1987), by gently rubbing the mucosal
surface of the cheek with a sterile tongue depressor. The cells were pooled, washed twice with HBSS (Paul, 1975) and harvested by centrifugation at 500 g for 10 min. Only freshly prepared cells were used in adherence assays.

**Adherence assay**

For the determinations recorded in Tables 1 and 2, exponentially growing yeast cells were inoculated at 10^5/ml into growth medium in the absence or presence (either 0.25 or 0.5 × MIC) of drug. Concentrations of drugs slightly lower than MIC values were used to permit yeast growth whilst retaining some of the cellular effects of the drugs. Cultures were grown in an orbital incubator at 37°C and shaking at 160 rev/min for either 60 min or 24 h (long- or short-term exposure to the drugs), harvested by centrifugation and washed twice in HBSS, then standardized following haemocytometer counting to 10^7 cells/ml of HBSS. Yeast cells prepared thus were used in the adherence determination described below. For the determinations recorded in Table 3, exponentially growing C. albicans KCCC 14172 and freshly isolated buccal epithelial cells were suspended individually (at 10^6 and 10^7 cells/ml in HBSS respectively) in the presence or absence of drug (0.5 × MIC). Cells were washed twice in HBSS and used in the adherence determination as below.

**Table 1. Effect of growth of Candida spp. in media containing octenidine on the adherence to buccal epithelial cells**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Treatment time (h)</th>
<th>Control</th>
<th>0.25 × MIC</th>
<th>0.5 × MIC</th>
<th>Inhibition of adherence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>ATCC 10231</td>
<td>24</td>
<td>607 ± 65</td>
<td>632 ± 28*</td>
<td>332 ± 32*</td>
</tr>
<tr>
<td></td>
<td>KCCC 14172</td>
<td>24</td>
<td>608 ± 42</td>
<td>367 ± 28*</td>
<td>269 ± 33*</td>
</tr>
<tr>
<td></td>
<td>C. tropicalis</td>
<td>24</td>
<td>374 ± 27</td>
<td>285 ± 18*</td>
<td>171 ± 16*</td>
</tr>
<tr>
<td></td>
<td>C. pseudotropicalis</td>
<td>24</td>
<td>284 ± 23</td>
<td>222 ± 16*</td>
<td>181 ± 13*</td>
</tr>
<tr>
<td>C. albicans</td>
<td>ATCC 10231</td>
<td>1</td>
<td>509 ± 25</td>
<td>386 ± 28*</td>
<td>346 ± 26*</td>
</tr>
<tr>
<td></td>
<td>KCCC 14172</td>
<td>1</td>
<td>551 ± 26</td>
<td>374 ± 27*</td>
<td>338 ± 26*</td>
</tr>
<tr>
<td></td>
<td>C. tropicalis</td>
<td>1</td>
<td>336 ± 18</td>
<td>290 ± 16</td>
<td>222 ± 16</td>
</tr>
<tr>
<td></td>
<td>C. pseudotropicalis</td>
<td>1</td>
<td>276 ± 24</td>
<td>217 ± 18</td>
<td>202 ± 16*</td>
</tr>
</tbody>
</table>

*Significant difference from control (p < 0.05)

**Table 2. Effect of growth of Candida spp. in media containing pirtenidine on the adherence to buccal epithelial cells**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Treatment time (h)</th>
<th>Control</th>
<th>0.25 × MIC</th>
<th>0.5 × MIC</th>
<th>Inhibition of adherence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>ATCC 10231</td>
<td>24</td>
<td>545 ± 26</td>
<td>402 ± 24*</td>
<td>311 ± 21*</td>
</tr>
<tr>
<td></td>
<td>KCCC 14172</td>
<td>24</td>
<td>523 ± 29</td>
<td>387 ± 30*</td>
<td>313 ± 25*</td>
</tr>
<tr>
<td></td>
<td>C. tropicalis</td>
<td>24</td>
<td>335 ± 26</td>
<td>275 ± 20*</td>
<td>201 ± 17*</td>
</tr>
<tr>
<td></td>
<td>C. pseudotropicalis</td>
<td>24</td>
<td>271 ± 22</td>
<td>193 ± 18*</td>
<td>164 ± 16*</td>
</tr>
<tr>
<td>C. albicans</td>
<td>ATCC 10231</td>
<td>1</td>
<td>415 ± 24</td>
<td>334 ± 24*</td>
<td>296 ± 23*</td>
</tr>
<tr>
<td></td>
<td>KCCC 14172</td>
<td>1</td>
<td>408 ± 27</td>
<td>323 ± 22*</td>
<td>290 ± 20*</td>
</tr>
<tr>
<td></td>
<td>C. tropicalis</td>
<td>1</td>
<td>299 ± 19</td>
<td>253 ± 20</td>
<td>229 ± 17*</td>
</tr>
<tr>
<td></td>
<td>C. pseudotropicalis</td>
<td>1</td>
<td>230 ± 17</td>
<td>219 ± 15</td>
<td>205 ± 16*</td>
</tr>
</tbody>
</table>

*Significant difference from control (p < 0.05)
Adherence of yeast to the epithelial cells was assayed using the technique described by Ghannoum et al. (1986). Two millilitres of each suspension (yeast and cells) were mixed in a sterile screw cap bottle. The mixture was shaken (160 rev/min and 37°C) for 2 h, then filtered through a 20 μm pore filter to remove non-adherent yeast cells. The epithelial cells on the filter were washed twice with 5 ml portions of HBSS and finally suspended in 5 ml of HBSS. Adherence was determined microscopically by counting the mean number of yeast cells adhering to 100 epithelial cells. Duplicate counts were made. Each assay was carried out in duplicate on separate occasions and a Student's t-test was used to evaluate the adherence values. A p-value of <0.05 was considered to be significant.

**Effects on germ-tube formation**

*C. albicans* KCCC 14172 cells grown with and without drugs were washed 3 times with distilled water. An inoculum from each preparation was added to tubes containing calf serum (Gibco, Paisley, Scotland) and incubated in a shaking water bath at 37°C. Samples were taken at intervals and added to an equal volume 1% glutaraldehyde in phosphate-buffered saline for fixation. Germ-tube formation was determined microscopically (Soll, Bedell and Brummer, 1981) Numbers of cells showing germ tubes/300 cells were recorded in triplicate Mean values (+ SE) were calculated and rounded to whole numbers.

**RESULTS**

Table 1 shows the effects of incubation of the yeast cells for a long (24 h) or a short (60 min) period in the presence of octenidine prior to the determination of adherence. Long exposure reduced adherence of all 4 yeast isolates to between 36 and 55% of the control value when using 0.5 x MIC, and to between 24 and 40% when using 0.25 x MIC. With the shorter exposure, 0.5 x MIC still produced significant inhibition with all 4 strains, although this was less than that achieved with the longer preincubation. With 0.25 x MIC, significant inhibition of adherence was observed with the 2 *C. albicans* strains but not with *C. tropicalis* or *C. pseudotropicalis*. A similar experiment was performed using pirtenidine (Table 2); this gave results that were qualitatively similar to those for octenidine although, in general, pirtenidine was less potent. The effects of pretreatment of yeast and buccal cells for 30 min with octenidine and pirtenidine before assay are presented in Table 3; this shows that reduction in adherence can be achieved by pretreatment of either partner. No significant differences could be found between pretreatment of either cell type or between the two drugs. Pretreatment of both partners resulted in a greater reduction in adherence compared to pretreatment of either partner, although the magnitude of the reduction was less than that which would have been expected from a combination of the independent effects on the two cell types.

Figure 2 compares the adherence of *C. albicans* from stationary phase and exponential phase and also compares the effects of preincubation with inclusion of octenidine and pirtenidine in the assay medium. It is clear that there is little or no difference between the adherence of cells from either stationary or exponential phases. In all cases, inclusion of drug in the assay medium inhibited adherence, but this was usually less than with preincubation.

Previous observations that germ tubes in *Candida albicans* adhere more strongly to buccal epithelial cells than do yeast blastospores (Kimball and Pearsall, 1980, Sobel et al., 1981) suggested that, with this species, blocking germination with the drugs might contribute to the inhibition of adherence. Table 4 shows that both drugs markedly inhibited germ-tube formation in *C. albicans* (KCCC 14172) even at sub-MIC concentrations.

**DISCUSSION**

Our findings clearly show that both octenidine and pirtenidine interfere with the adherence of *Candida* spp. to buccal epithelial cells in vitro. This was so for both short- and long-term preincubation with the drugs, although the longer term was generally more effective. Pretreatment of either buccal or yeast cells or both was effective, as was inclusion of the drugs during the adherence assay. There was no significant difference between the adherence potential of yeast from exponential and stationary phases. The mechanisms responsible for inhibition of adherence are still to be determined but these could include alterations to cell surface features, which could mask the adhesins present on the yeast or receptors present on the buccal cells. Chlorhexidine, which interferes with adherence of *C. albicans* to buccal epithelial cells (Tobgi et al., 1987), affects its outer envelope (Bobichon and Bouchet, 1987); octenidine and pirtenidine disrupt the ultrastructural organization of its cell wall (Ghannoum et al., 1989).

Another possibility for the *C. albicans* strains is that...
Fig. 2. Effect of octendine and piritendine on adherence of yeast cells from exponential and stationary phase to buccal epithelial cells. Exponential phase *C. albicans* KCC14172 were inoculated at 10^6/ml into growth medium with or without octendine or piritendine. These were grown to exponential phase (12 h) or stationary phase (24 h) at 37°C. Cells were harvested, washed twice in HBSS and standardized. Adherence was assayed either in the presence or absence of the drugs (0.5 x MIC). A—Yeast grown for 24 or 12 h without drug. B—Yeast grown for 24 or 12 h in the presence of drug. C—Drug included in assay medium. Arrows indicate SE values.

Table 4 Effect of octendine and piritendine on germ-tube formation in *C. albicans*

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Control</th>
<th>0.25 x MIC</th>
<th>0.5 x MIC</th>
<th>MIC</th>
<th>0.25 x MIC</th>
<th>0.5 x MIC</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>62 ± 4</td>
<td>25 ± 4</td>
<td>11 ± 1</td>
<td>5 ± 1</td>
<td>37 ± 4</td>
<td>22 ± 3</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>120</td>
<td>87 ± 5</td>
<td>37 ± 2</td>
<td>15 ± 4</td>
<td>6 ± 1</td>
<td>56 ± 4</td>
<td>24 ± 3</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>180</td>
<td>98 ± 1</td>
<td>44 ± 2</td>
<td>15 ± 1</td>
<td>6 ± 1</td>
<td>74 ± 3</td>
<td>30 ± 4</td>
<td>22 ± 3</td>
</tr>
</tbody>
</table>

the drugs might affect germ-tube formation, thus influencing adhesion indirectly (Sobel et al., 1981). We have demonstrated that both octendine and piritendine, at concentrations well below those required to inhibit growth completely, significantly reduce germ-tube formation. The combined antifungal and anti-adhesion properties of these drugs and the fact that they have effects at concentrations less than MIC values, make them good candidates for further investigation of their prophylactic potential against candidal infections.

Acknowledgement—This work was supported by Kuwait University Research Council Grant No S0038

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Drugs and *Candida* adhesion to buccal cells


