ADHESION OF *Candida albicans* TO HOST CELLS IN CULTURE

by

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To my husband, Mahomed

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INTRODUCTION

Candidiasis is a disease caused mainly by the fungus, *Candida albicans*, and to a lesser extent by other *Candida* spp. such as *C. tropicalis* and *C. parapsilosis*. The disease may present itself as superficial candidiasis, of which the most common form is superficial lesions of mucosae referred to as thrush. Alternatively, *C. albicans* may cause serious disseminated (systemic) infection. *Candida* is a dimorphic opportunistic pathogen that resides as a commensal of vertebrate hosts, but is capable of infecting almost every type of tissue of the body (Shepherd *et al.*, 1985). Candidiasis does not normally occur in healthy persons and is difficult to eradicate in individuals with poor defence mechanisms (Shepherd, 1982).

Although superficial candidiasis is not a serious disease, it cannot be ignored in view of its persistent nature. Since the 1960's an increase in the use of antibiotics, immunosuppressants and hormones has resulted in increased incidence of the disease. Candidal vulvovaginitis affects up to 40% of the adult female population (Friedrich, 1988) and is recurrent in about 20% of these patients (Segal, 1987). Although oral candidiasis usually affects neonates, probably due to a lack of defence mechanisms, it is found to occur with increasing frequency in adults undergoing extensive antibiotic or immunosuppressive therapy. An estimated 65% of people wearing dentures

suffer from denture stomatitis which is caused by candidal infection (Samaranayake, 1986). Chronic and recurrent manifestations of candidiasis are particularly resistant to antifungal therapy. The host factors predisposing to primary candidiasis are largely unclear. There has to be some change in the host environment that stimulates yeast colonization of host tissue, which is the first step in its transition from commensal to pathogen.

The main host factors implicated in increased susceptibility to candidiasis are suppression of the immune response, a decrease in competing microbial flora and alterations in tissue cytology. One of the virulence factors of *C. albicans* is presumably its ability to adhere to host tissue. Since strains of *C. albicans* are capable of infecting tissue from different anatomical sites, the organism must possess remarkable genetic adaptability (Soll, 1988). Once the yeast cell is able to adhere, it then changes to a mycelial form of growth which is more suitable for tissue invasion and resisting host defence mechanisms (Shepherd *et al.*, 1985). The organism produces enzymes to destroy host tissue, facilitating invasion and releasing nutrients. The infection may establish itself as a disease if the host defence is unable to eliminate the organism. Thus, it appears that preventing the organism from adhering, would be the most suitable method to resolve the problem.

Studies on the adherence of *C. albicans* to host cells have concentrated on the use of adherence inhibition studies (Collins-Lech *et al.*, 1984; Critchley & Douglas, 1987a; Sandin, 1987; Segal *et al.*, 1982; Sobel *et al.*, 1982) or manipulation of the yeast growth conditions (McCourtie & Douglas, 1984) to investigate the mechanism(s) of adhesion. Blocking experiments usually entailed the use of sugars or lectins to determine the nature of the adhesin and/or receptor. The adhesin is considered to reside on the yeast cell surface while the receptor is associated with the host cell membrane. It was suggested that the adhesin is a fibrillar component comprising mannoprotein (Tronchin *et al.*, 1984). The nature of the host cell receptor is unclear. However, there is some indication that glycosides comprise at least part of the receptor (Critchley & Douglas, 1987b). In the past decade, much information has been collected in this area of study. However, the information is largely contradictory, a fact which has been attributed to the differences in the assay methods and parameters that were employed. Most of the information was pertaining to the use of exfoliated host cells obtained from donors, in particular buccal epithelial cells (BEC). The common method to quantify adhesion was by microscopic observation (Collins-Lech *et al.*, 1984; Critchley & Douglas, 1987a; Sandin, 1987).

In view of the need for a controlled host model for the interaction this study investigated the possibility of using normal primary vaginal epithelium for that purpose. A method to culture the tissue, which was exacting in its requirements, was devised. The gentlest possible method to dissociate the cells was determined. Growth conditions conducive to yeast cell formation were employed. The adherence of yeast cells to dissociated epithelial cells was quantified by particle size determination and compared to the microscopic observations. The effects of mannose and concanavalin A (conA) on adherence were determined. Mannose and its complementary lectin were chosen for the adherence inhibition study because of the contradictory information on their involvement in adhesion. On the same basis, *Escherichia coli*, was used to determine whether this organism had any positive effect on adherence. A buccal cell line and non-cultured cells from the rat were used for a comparison of the results with cultured vaginal epithelial cells (VEC). In addition, the host-pathogen interaction was observed ultrastructurally. The lack of previous studies concerning the latter, with reference to cultured vaginal tissue, raised the question as to whether the observations were similar to that of *in vivo* studies using intact epithelium or scrapings of cells. The advantages and disadvantages of the methods employed are discussed. It should be noted that the terms "adhesion" and "adherence" are used interchangeably.

I. LITERATURE REVIEW

A) THE ROLE OF ADHESION IN PATHOGENESIS

The adhesion of a pathogenic microorganism to host tissue is a prerequisite for colonization and infection, since it allows for the establishment of a microorganism in an otherwise hostile environment. Bacterial systems have received much attention in this regard (Finlay & Falkow, 1989). In the past decade, studies have also focussed on the importance of adherence in candidiasis.

1. Species and Strain Differences

The ability of *Candida* species to adhere to host tissue has been correlated with their capacity for causing infection. King *et al.* (1980) observed that *C. albicans* was most adherent to VEC and BEC compared to six other *Candida* species. *C. tropicalis* and *C. stellatoidea* were moderately adherent, followed by *C. parapsilosis*, which was poorly adherent. The other species were non-adherent. The authors suggested that the ability of *Candida* species to adhere may be related to their colonizing capabilities. In Table 1 (Ray *et al.*, 1984) the adherence of six *Candida* species to corneocytes and BEC was compared to their status as murine cutaneous pathogens. *C. albicans* and *C. stellatoidea* were the most adherent and pathogenic species.

Differences were observed in the ability of certain *C. albicans* strains to adhere to BEC (McCourtie & Douglas, 1984). Seven strains isolated from active infections (I

TABLE I. Comparison of epithelial cell adherence and pathogenicity in experimental murine cutaneous candidiasis (Ray *et al.*, 1984)

	ADHERENCE		PATHOGENICITY FOR MURINE EPIDERMIS
	CORNEOCYTES	BUCCAL CELLS	
C. albicans	+	+	+
C. stellatoidea	+	+	+
C. tropicalis	-	-	-
C. parapsilosis	±	-	-
C. guilliermondii	-	-	-
C. krusei	-	-	-

strains) and two strains from asymptomatic carriers (C strains) were grown in medium containing high concentrations of either galactose or sucrose as compared to growth in medium with a low glucose concentration. The adherence and pathogenicity of I strains was 5 times greater for growth in sucrose and 5 to 24 times greater for galactose. It was suggested that I strains react with sugars in the microenvironment and are capable of

modifying their cell surface composition, resulting in enhanced adherence and virulence, as compared to C strains. In a study by Critchley & Douglas (1985), species and strain differences were observed for the adherence of *Candida* to BEC, using the same method described by McCourtie & Douglas (1984), but only testing the effect of galactose. *C. albicans* was most adherent followed by one strain of *C. tropicalis*. A second strain of the latter yeast and four other *Candida* species, as well as the yeast, *Saccharomyces cereviseae* did not show increased adherence.

Forty-one isolates of *C. albicans* from vaginitis patients were tested for their adherence capacity compared to thirty-six isolates from asymptomatic carriers. The number of adherent yeasts and adherent VEC, as determined per 100 cells, was significantly higher for isolates from infected patients (Segal *et al.*, 1984). A correlation was observed between phospholipase A activity, adherence to BEC and virulence, for 4 species of *Candida* (Barrett-Bee *et al.*, 1985). Two pathogenic isolates displayed high enzyme activity and adherence. Of the remaining isolates which were obtained from a single individual, one showed low enzyme activity, poor adherence and mild virulence while the other had much higher enzyme activity and adherence along with moderate pathogenicity.

2. Host Susceptibility

Host epithelial cells differ in their capacity to bind to *Candida*. The variation in adherence of *C. albicans* to mucosal cells from different anatomic sites is shown in Fig. 1 (Sandin *et al.*, 1987). It should be noted that *C. albicans* was most adherent to BEC, followed by VEC and least adherent to urinary epithelial cells. The latter

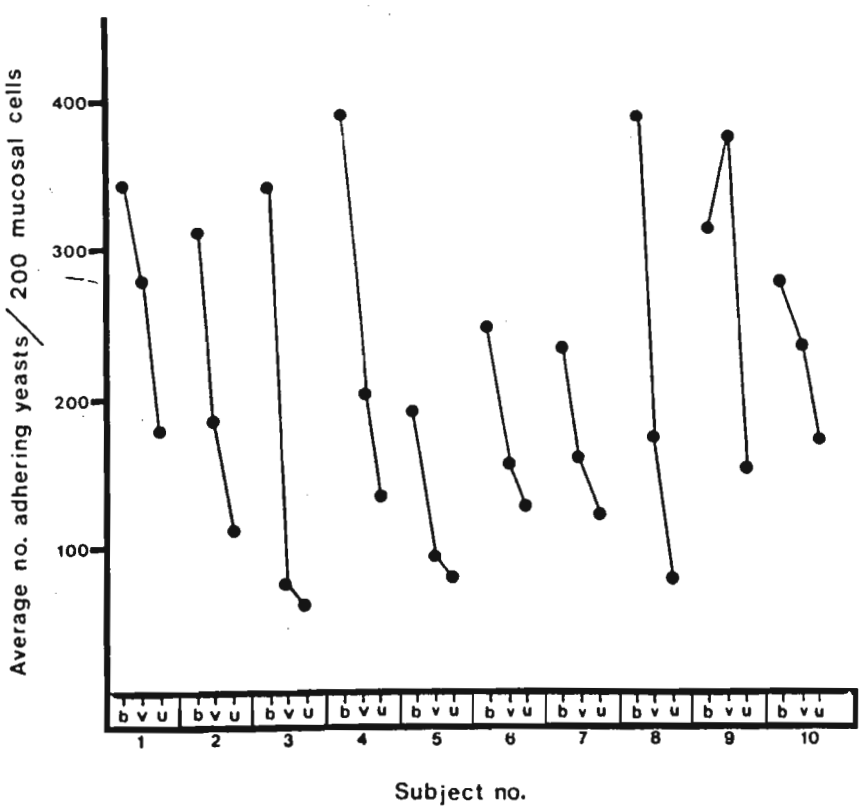

FIG. 1. Yeast adherence to mucosal cells from various body sites, collected from ten subjects on one date. The symbols representing the mucosal cell site of origin are as follows: b for buccal, v for vaginal and u for urinary tract (Sandin *et al.*, 1987)

cells appeared much smaller than BEC and VEC, and may be the reason that they were least adherent.

(a) Buccal epithelial cells (BEC)

In addition to differences between mucosal cells from different anatomical sites, the effect of various collection dates and influence of donor on candidal adherence to BEC was determined. According to Collins-Lech *et al.* (1984) and Sandin *et al.* (1987) respectively, significant differences were observed in the adherence of *Candida* to BEC collected on different dates (Fig. 2) as well as BEC from 24 different donors (Fig. 3). This was contrary to observations by Cox (1983), who did not find any significant donor difference or day to day variation in BEC from healthy adults. Similarly, Botta (1981) did not find any difference in day to day variation in the *Candida*-binding capacity of pharyngeal cells. Iron and certain vitamin deficiencies may play a role in increasing the host predisposition to candidiasis, possibly by causing increased keratinization of host epithelium (Samaranayake, 1986).

(b) Vaginal epithelial cells (VEC)

King *et al.* (1980) found that donor and daily variations existed in the adherence of *Candida* to VEC. Variation in candidal adherence to VEC during different weeks of the menstrual cycle was observed. Botta (1981) reported

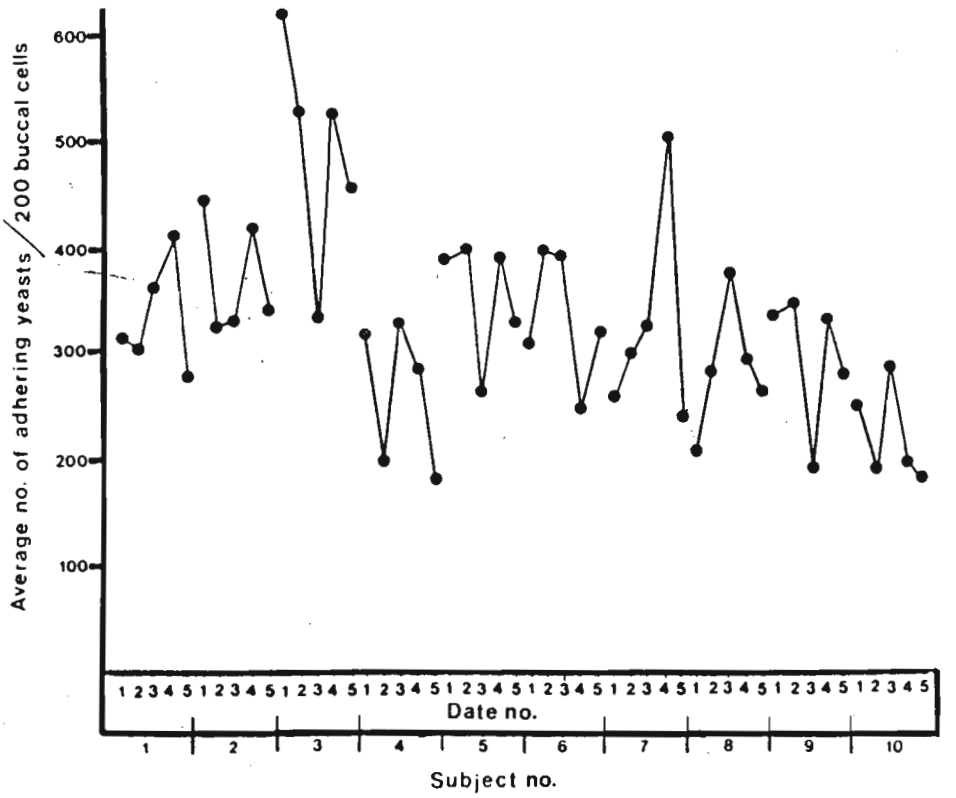

FIG. 2. Variation in yeast adherence to buccal mucosal cells from ten subjects on five collection dates (Sandin *et al.*, 1987)

highest adherence in the third week while Segal *et al.* (1984) observed increased adherence during the first and fourth weeks of the cycle (Fig. 4). The predominant VEC during the first and fourth weeks was found to be the intermediate cell type. In an experiment by Kalo & Segal (1988) the intermediate VEC was found to be significantly more adherent than the superficial cell type. A correlation was observed between the susceptibility of different physiological groups of women to candidiasis and the *Candida*-binding capacity of their VEC (Table II).

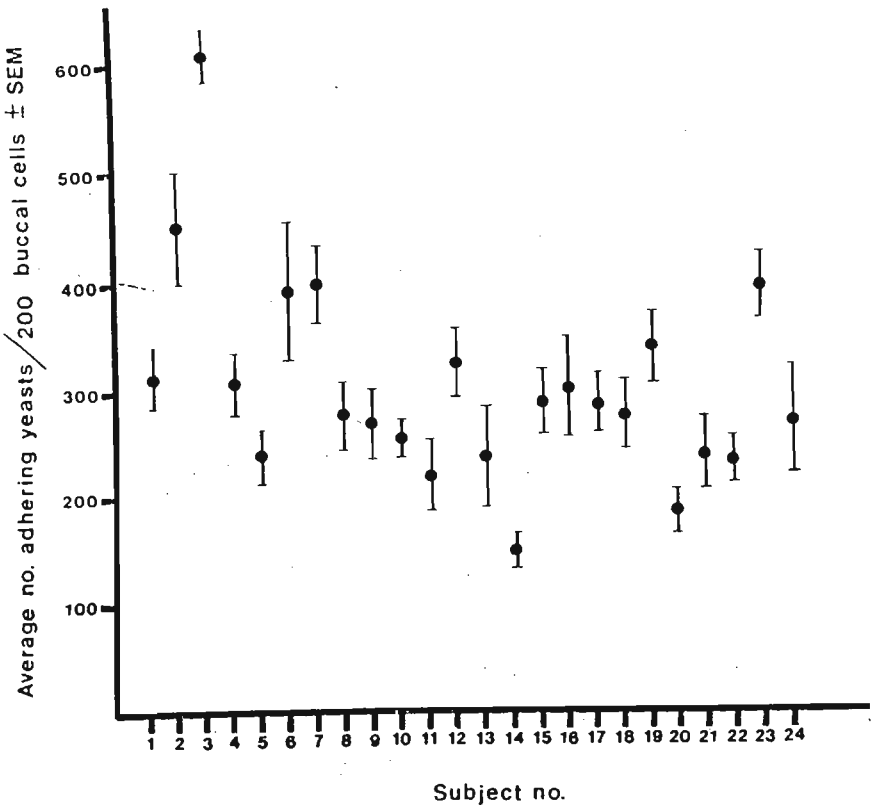

FIG. 3. Variation in the adherence of *C. albicans* to buccal mucosal cells from different subjects. Experiments using buccal cells from each subject were run in triplicate (Sandin *et al.*, 1987)

Candidal adherence to VEC was highest for pregnant diabetic women followed by pregnant women and diabetic women. Postmenopausal diabetic women had VEC that showed increased adherence. These conditions were associated with relatively low Karyopycnotic Index (KPI) values, which indicated increased numbers of intermediate cells. KPI was measured by the percentage of superficial cells, indicating hormonal state in fertile females. Maturation Index (MI) was measured as for KPI, but for menopausal

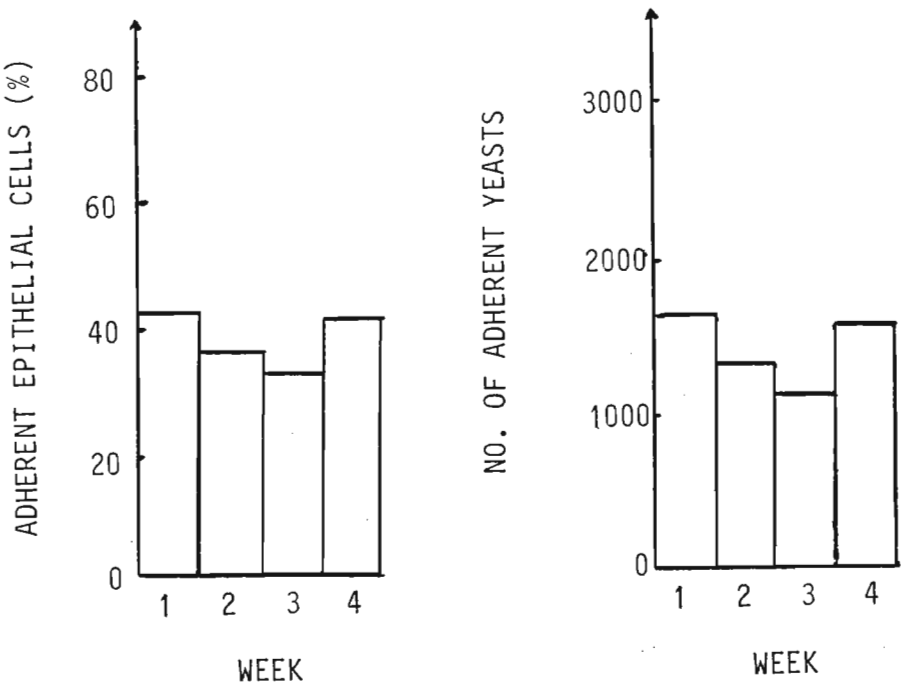

FIG. 4. Comparison of adhesive interactions between *C. albicans* and vaginal epithelial cells from women at various weeks of the menstrual cycle (Segal *et al.*, 1984)

females. Cells from postmenopausal women displayed decreased adherence, in spite of a low KPI value. The authors suggested this to be a result of the predominant parabasal cell type which has a smaller surface area for attachment compared to the other vaginal cell types. There was no significant difference in the adherence of *Candida* to VEC from women using oral contraceptives (Segal *et al.*, 1984). These results were not in keeping with the observations of Galask (1988), who found that candidal

TABLE II. *C. albicans* adherence *in vitro* in relation to KPI/MI values (Segal *et al.*, 1984)

GROUP		NO. OF WOMEN	ADHERENCE (MEAN VALUES)					
			ADHERENT EPITHELIAL CELLS (%)	TOTAL NO. ADHERENT YEASTS	KPI/MI (MEAN VALUES)	S	I	P
I[a]	Fertility Control	75	37	1362.4	51.2			
II	Using Oral Contraceptives	71	40	1368.7	49.5			
III	Diabetics	27	51	1929.3	28.7			
IV	Pregnant	65	51	1523.3	16.5			
V	Pregnant diabetic	28	59	2205.5	15.8			
VI[b]	Postmenopause (Control)	48	32	1017.9	19.0	19.0	39.0	41.7
VII	Diabetics	33	48	1638.5	11.7	11.7	73.4	14.7

[a]Fertility age (18-45 years), groups I-V;

[b]Postmenopause, groups VI-VII

S - superficial epithelial cells; I - intermediate epithelial cells; P - parabasal epithelial cells; KPI - Karyopycnotic Index; MI - Maturation Index

colonization was 7% higher in patients using oral contraceptives compared to those who did not. The difference may be attributed to the types of oral contraceptive used (Ryley, 1986).

The effect of hormones on the adherence of *Candida* to VEC or HeLa cells pretreated with sex hormones is described in Figs. 5 and 6, respectively (Kalo & Segal, 1988). The VEC

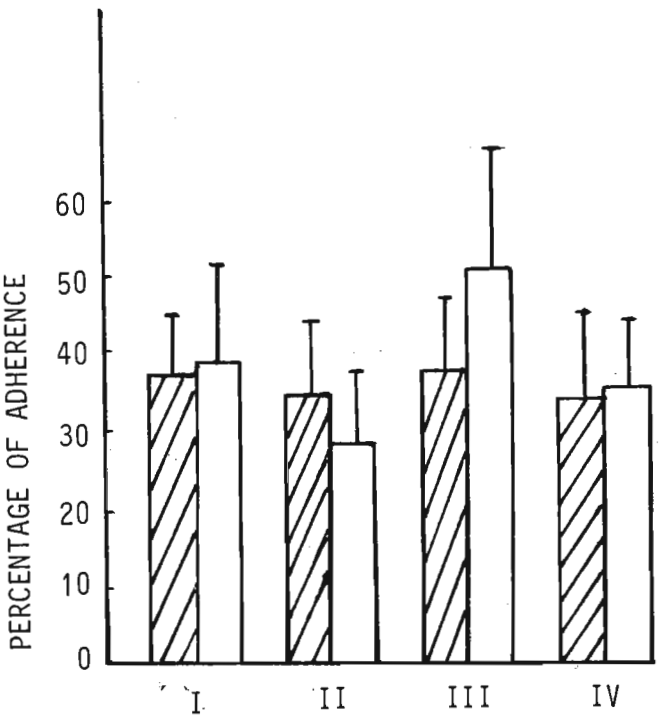

FIG. 5. Effect of sex hormones on adherence *in vitro* of *C. albicans* to exfoliated human VEC. Mean values from six experiments, each in duplicate. I, 17-ß-oestradiol (1 $ng.ml^{-1}$); II, oestriol (1 $ng.ml^{-1}$); III, progesterone (20 $ng.ml^{-1}$); IV, testosterone (20 $ng.ml^{-1}$); ▨, control; □, with hormone (Kalo & Segal, 1988)

and HeLa cells were pretreated for 18 h and 24 h, respectively with either 17-ß-oestradiol, oestriol, progesterone or testosterone. The highest increase in adherence was obtained with testosterone pretreatment of

HeLa cells. A significant increase was obtained with progesterone pretreatment of VEC and HeLa cells while VEC treatment with oestriol resulted in decreased adherence. In contrast, *in vivo* studies indicated that rat vaginal colonization with *Candida* could only be maintained when animals were in oestrus or pseudoestrus suggesting the

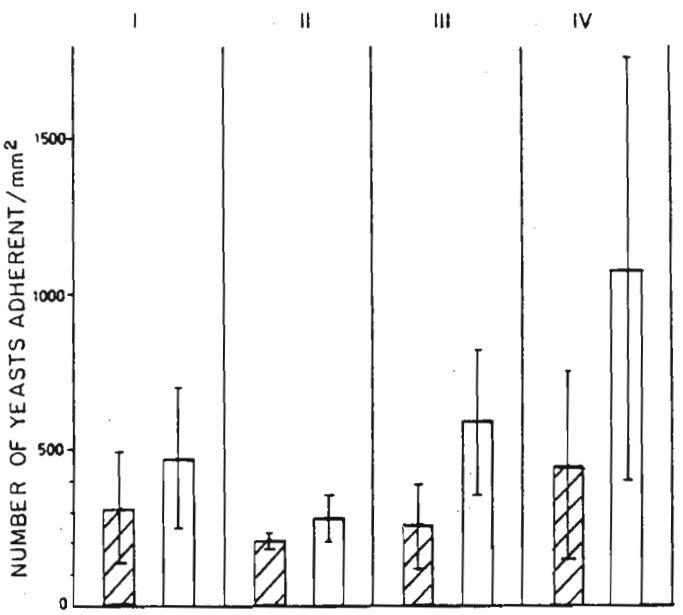

FIG. 6. Effect of sex hormones on adherence *in vitro* of *C. albicans* to HeLa cells. HeLa cells were exposed to the sex hormones 17-ß-oestradiol (1 $ng.ml^{-1}$), oestriol (1 $ng.ml^{-1}$), progesterone (20 $ng.ml^{-1}$), and testosterone (20 $ng.ml^{-1}$) for 24 h. I, 17-ß-oestradiol; II, oestriol III, progesterone; IV, testosterone; ▨, control; □, with hormone (Kalo & Segal, 1988)

importance of oestrogen and keratinization of epithelium in vaginal candidiasis (Kinsman & Collard, 1986; Larsen & Galask, 1984). The hormonal status *in vivo* affects not only cell type and proliferation, but also the local immune response and microflora. This complex situation may be the reason that discrepancies in results of the effect of hormones on VEC *in vivo* and *in vitro* were observed.

3. Effect of Bacteria

Bacteria, in the microenvironment, may interfere with yeast adherence by competing for binding sites on epithelium, by modification of binding sites or by the production of metabolites (Larsen *et al.*, 1977; Sandin *et al.*, 1987). According to Cox (1983), a decrease in the competing bacterial flora brought about by antibiotic treatment resulted in increased candidal adherence to BEC, which was attributed to an increase in salivary glucose owing to decreased bacterial utilization.

Kennedy & Volz (1985) reduced the numbers of hamster intestinal bacteria by antibiotic treatment. As a result candidal colonization increased and dissemination of the yeast occurred from the gut. The authors suggested two possible mechanisms whereby indigenous microflora of the gut inhibit *C. albicans* colonization and dissemination, viz.:

- the size of the yeast population is suppressed by bacteria; or
- bacteria may form thick layers in the mucus gel and thereby provide a physical barrier to the yeasts.

The attachment of *C. albicans* to epithelial cells decreased when cells were precoated with vaginal isolates of lactobacilli (Sobel *et al.*, 1981). In contrast, Larsen & Galask (1984) found that the increased presence of bacteria, in the rat vagina, did not prevent yeast colonization. Centeno *et al.* (1983) observed that pretreatment of uroepithelial cells with piliated *E. coli* or *Klebsiella pneumoniae* resulted in significantly increased adherence of *C. albicans* to the epithelial cells compared to pretreatment with non-piliated strains. Similarly, the adherence of *C. albicans* to HeLa monolayers was significantly increased by pretreatment of the tissue with *E. coli* (Makrides & MacFarlane, 1983) which led the authors to concur with the proposal by Centeno *et al.* (1983) that bacteria possessing mannose-sensitive pili may attach to epithelium as well as bind to mannan-containing moieties on the yeast surface, thereby facilitating adherence.

B) MECHANISMS OF ADHESION

In general, cells normally possess a net negative surface repulsion by electrostatic forces. Van der Waals forces

are attractive and create a net force of attraction at particular distances of separation (Bell, 1978). Initially a weak interaction occurs which may be reversed easily and is possibly non-specific in nature. As the cells are brought even closer, a stronger irreversible bond is formed. The interaction is stereospecific and mediated by adhesins and receptors which reside on the cell surface of the microbe and host, respectively (Keusch, 1979; Sobel & Obedeanu, 1984).

A decrease in the radius of the interacting components results in a net increase in attraction and explains why bacterial pili are well suited to mediate adhesion. Unlike bacteria, yeasts are much larger and lack motility, thereby creating conditions not very conducive to adhesion. The *C. albicans* seems to have overcome this problem by producing a fibrillar outer layer on the cell surface which is probably involved in mediating adherence of the organism to host cells (Douglas, 1987).

1. *C. albicans* Adhesin

(a) Yeast cell surface

The cell wall of *C. albicans* yeast and germ tube-forming cells comprised 48 to 60% glucan, 20 to 23% mannoprotein, 3 to 6% protein, 0,6 to 2,7% chitin and 2% lipid (Sullivan *et al.*, 1983). According to Poulain *et al.* (1985) the cell wall may be divided into 8 layers based on ultrastructural evidence. These authors suggested that

the outermost layers, designated 0 and 1, are involved in adherence and host tissue lysis while layers 2 to 4 comprise a mannan or mannoprotein matrix which is of immunogenic importance. Layers 5 to 7 contained mostly glucan while layer 8, which was also referred to as the periplasmic space, comprised chitin and proteins. The number of wall layers observed depended on growth conditions and cytochemical methods. Transmission electron microscopy (TEM) revealed the presence of 3 (Garcia-Tamayo *et al.*, 1982) and 5 layers (Shepherd *et al.*, 1985). Marrie & Costerton (1981) observed a ruthenium red positive matrix which appeared to mediate adherence of *C. albicans* to BEC. Pugh & Cawson (1978), using scanning electron microscopy (SEM), observed a flocculent layer of varying thickness on the surface of *C. albicans* yeasts. They also found that the amount of extracellular material produced increased with the age of the culture. Preparations for TEM revealed strong staining of the surface layer with ruthenium red, as well as granular extracellular material.

The outermost layer, associated with adherence, was fibrillar in appearance. This layer, comprising thin fibrils perpendicular to the yeast surface (Tronchin *et al.*, 1984), was produced in response to high concentrations of sugars, notably galactose and sucrose, while apparently in the stationary phase of growth (McCourtie & Douglas, 1984). The type of sugar and the

concentration employed determined, within a culture, the proportion of *C. albicans* yeasts on which the adhesin was fully expressed. A homogeneous population of cells bearing optimal amounts of fibrillar adhesin was detected with a high concentration of galactose in the growth medium. The adhesin was unequally distributed in a heterogeneous population of yeasts grown in medium containing either high sucrose or low glucose concentrations (McCourtie & Douglas, 1985a). Cytochemical methods employing conA and mannosyl-ferritin indicated that the fibrillar component comprised mannoprotein (Tronchin *et al.*, 1981). This was in agreement with agglutination studies using conA (Cassone *et al.*, 1978). Prolonged yeast incubation resulted in the release of fibrils into the culture medium (Critchley & Douglas, 1985).

(b) Chemical composition

The adhesin of *C. albicans* was suggested to be, at least in part, mannoprotein in nature. Sandin *et al.* (1982) found that pretreatment of BEC with conA decreased adherence by 83%, while the addition of mannose to the interacted mixture resulted in a 53% reduction. Pretreatment of host cells with mannose resulted in a 30% and 70% decrease in candidal adherence to uroepithelial and buccal cells, respectively (Centeno *et al.*, 1983). Although adherence to vaginal cells was not affected by mannose, yeast mannan or mannoprotein extracted from *C.*

albicans, Lee & King (1983) found that proteases and, to a lesser extent, reducing agents were effective in decreasing adherence. The authors suggested that the adhesin of *Candida* is a glycoprotein. The addition of conA to a HeLa monolayer resulted in a 17% increase in adherence of *C. albicans* to the tissue (Makrides & MacFarlane, 1983). According to Ray *et al.*(1984), the addition of yeast mannan or conA to the interacted mixture did not significantly affect adherence of *C. albicans* to BEC or corneocytes. However, at the 1 h and 3 h time points, it was noted that adherence to BEC was reduced and slightly increased by mannan, respectively (Fig. 7). In a relatively recent study by Sandin (1987), it was observed that the addition of conA, at 10 min intervals, to BEC interacted with *C. albicans* resulted in significantly decreased adherence up to a certain time after which adherence was unaffected (Fig. 8).

McCourtie & Douglas (1985b) were successful in blocking adherence of *C. albicans* to BEC using extracellular polymeric material (EP), mannoprotein in nature, from the culture supernatant. *C. albicans* yeasts, grown in medium containing either a high concentration of galactose, sucrose or low concentration of glucose, produced EP after 5 d of growth. As expected, galactose-grown yeasts were most adherent and produced most EP, followed by sucrose- and glucose-grown yeasts, in

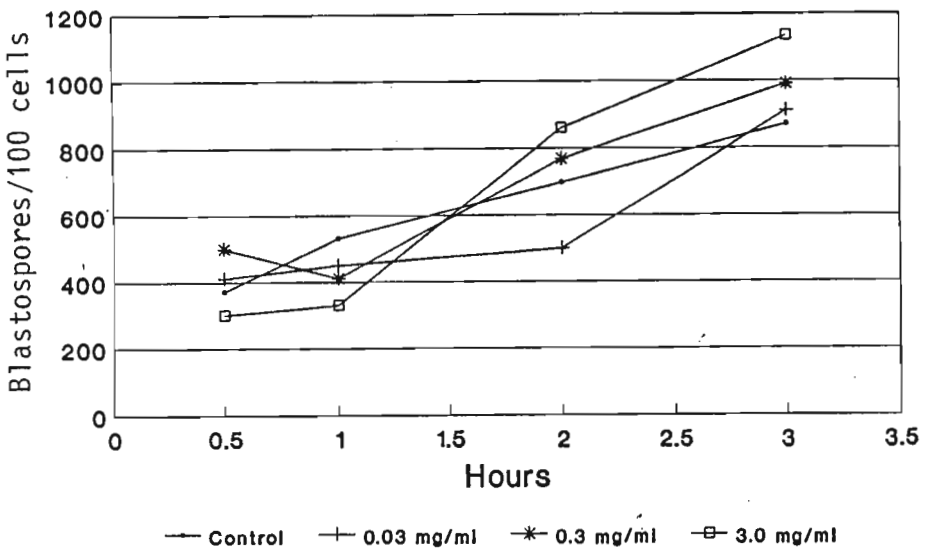

FIG. 7. Adherence of *C. albicans* to human buccal epithelial cells in the presence of graded concentrations of mannan over time (Ray *et al.*, 1984)

that order. However, the chemical composition of EP was similar for the different carbon sources (Table III). The EP comprised 65 to 82% mannose with some glucose, 7% protein, 1,5% glucosamine and 0,5% phosphorus. Although EP was immunologically identical, an increased number of antigenic determinants was present in EP of galactose-grown yeasts compared to the other carbon sources. Some strains of *C. albicans* displayed antigenic differences in their EP. The adherence of *C. albicans* to BEC pretreated with EP, was reduced (Table IV). The EP

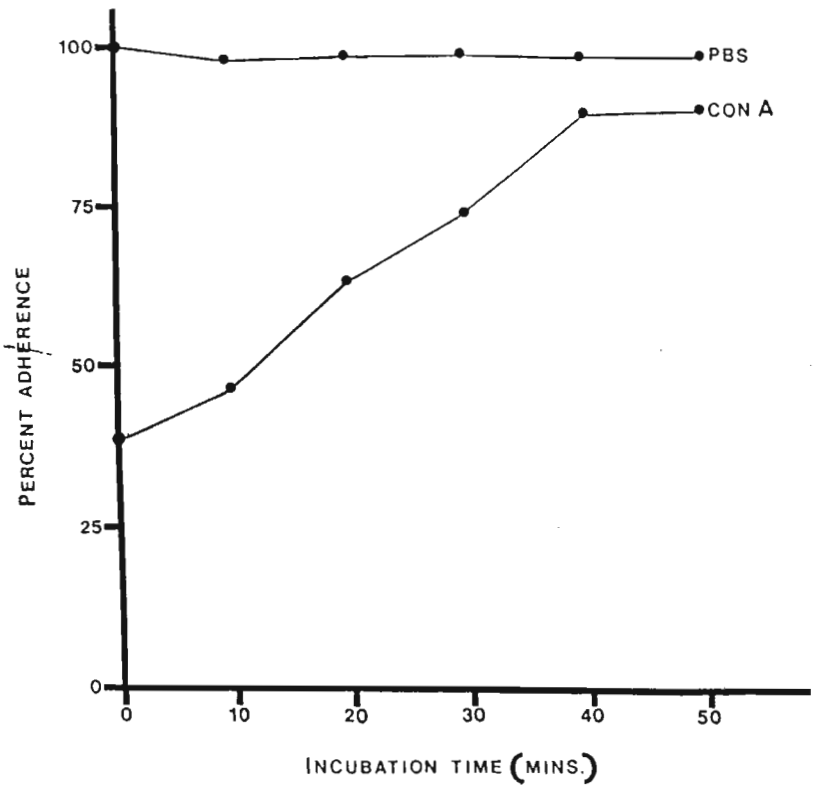

FIG. 8. Effect on adhesion of the addition of conA or PBS to incubating cells, as a function of time. 100% adherence = 307 YC/200 mucosal cells (Sandin, 1987)

from strain GDH2346 inhibited adherence for the same yeast strain by 30% and 50% for EP from medium containing sucrose or galactose carbon source, respectively. Similarly, a 50% reduction was observed for EP from strain GDH2023 grown in galactose-containing medium when tested against the same strain. Adherence was unaffected when this EP was tested against yeast strain GDH2346, emphasizing the strain difference in the nature of EP produced by *C. albicans*. Critchley & Douglas (1987a)

TABLE III. Yield of EP obtained from culture supernatants of *C. albicans* GDH2346. Results represent means ± SE for 3-7 independent experiments. Yield is expressed as a percentage of the combined dry weight of yeast cells and EP. Probability values (P) compare with those from corresponding cultures grown in medium containing 50 mM-glucose (McCourtie & Douglas, 1985b)

CARBON SOURCE	INCUBATION PERIOD (d)	DRY WT OF YEASTS (g)	DRY WT OF EP (mg)	PERCENTAGE YIELD OF EP	P
Glucose	1	1.67 ± 0.19	75 ± 12	4.3 ± 0.5	
Sucrose	1	2.69 ± 0.59	137 ± 31	4.8 ± 0.2	NS
Galactose	1	2.35 ± 0.44	137 ± 20	5.7 ± 0.3	< 0.05
Glucose	5	1.94 ± 0.19	92 ± 20	4.4 ± 0.8	
Sucrose	5	1.87 ± 0.07	221 ± 41	10.5 ± 1.8	< 0.025
Galactose	5	2.72 ± 0.46	576 ± 115	17.1 ± 1.5	< 0.001

NS - Not significant

purified the mannoprotein adhesin contained in EP. The purified adhesin was 30 times more efficient in reducing adherence to BEC compared to EP. The EP was partially or totally incapable of inhibiting adherence when pretreated

with heat, reducing agents or proteolytic enzymes. Since pretreatment of EP with sodium periodate or α-mannosidase did not have much effect, the authors suggested that the protein, rather than carbohydrate component of the adhesin, was involved to a large extent in mediating adherence to BEC.

Mannoprotein glycosylation was inhibited by the addition of the antibiotic tunicamycin to *C. albicans* grown in medium containing a high concentration of galactose (Douglas & McCourtie, 1983). Synthesis of the fibrillar outer wall layer was inhibited and decreased adherence of yeasts to BEC occurred compared to low concentration glucose-grown control yeasts (Table V). The adherence of *C. albicans* to VEC was increased by the addition of mannose (Reinhart *et al.*, 1985) while in other studies mannose had no significant effect on adherence to VEC (Segal *et al.*, 1982), BEC and corneocytes (Collins-Lech *et al.*, 1984). However, in the latter two studies significant inhibition of adherence was obtained by the addition of amino sugars to the interacted cells. The inhibitory amino sugars were glucosamine, galactosamine and mannosamine (Collins-Lech *et al.*, 1984) or N-Ac-glucosamine, chitin, its hydrolysate derivative and to a lesser extent glucosamine and mannosamine (Segal *et al.*, 1982). Sobel *et al.* (1981) found that L-fucose inhibited the adherence of *C. albicans* to VEC.

TABLE IV. Effect of pretreatment of buccal epithelial cells with EP on the subsequent adhesion of *C. albicans* GDH2346 and GDH2023 (McCourtie & Douglas, 1985b)

STRAIN	CARBON SOURCE IN GROWTH MEDIUM	MEAN NO. (± SE) OF ADHERENT YEASTS PER 100 EPITHELIAL CELLS PRETREATED WITH: PBS	EP	RELATIVE ADHERENCE*	P†
		(a) EP from strain GDH2346			
GDH2346	Glucose	114 ± 5	104 ± 6	0.9	NS
GDH2346	Sucrose	387 ± 17	292 ± 18	0.7	< 0.01
GDH2346	Galactose	847 ± 47	451 ± 27	0.5	< 0.001
		(b) EP from strain GDH2023			
GDH2023	Galactose	871 ± 34	419 ± 38	0.5	< 0.001
GDH2346	Galactose	692 ± 23	695 ± 33	1.0	NS

*Adherence to EP-treated epithelial cells compared with that to PBS-treated cells

†Probability values comparing adherence to EP-treated epithelial cells with that to PBS-treated cells. NS - Not significant

TABLE V. Effect of tunicamycin treatment on the adherence of *C. albicans* to buccal epithelial cells (Douglas & McCourtie, 1983)

CARBON SOURCE IN GROWTH MEDIUM	MEAN NUMBER OF ADHERENT YEASTS PER 100 EPITHELIAL CELL ± SE	
	TUNICAMYCIN - TREATED	UNTREATED
Glucose, 50 mM	102 ± 9	93 ± 8
Galactose, 500 mM	240 ± 28	630 ± 28

According to Ghannoum *et al.* (1986), certain lipids, viz., phospholipids, sterols and steryl esters may be involved, in addition to sugars, in the adherence of *C. albicans* to BEC. Lipid extracts from *C. albicans* and *C. tropicalis* were unable to affect adherence of *C. pseudotropicalis*. However lipid extracts of the latter organism were capable of reducing adherence of the former two yeasts.

2. Host Cell Receptor

The animal plasma membrane comprises lipids and proteins, both of which are mostly glycosylated. Glycolipids and integral glycoproteins have the carbohydrate component exposed on the cell surface. Peripheral glycoproteins are bound to the plasma membrane by weak ionic or hydrogen

bonds (Bell, 1978). The layer referred to as the "cell coat" contains carbohydrate, occurs on the surface and is closely associated with the plasma membrane. D-glucose is present in membrane glycolipids but not glycoproteins. The latter contain D-mannose, D-galactose, L-fucose, N-Ac-D-glucosamine, N-Ac-D-galactosamine and sialic acid (Douglas, 1987).

Considering the possibility that a sugar, being studied in adherence inhibition tests, represents the adhesin as well as the receptor, the most likely receptor would be D-mannose (Centeno *et al.*, 1983; Sandin *et al.*, 1982), L-fucose (Sobel *et al.*, 1981) or amino sugars (Collins-Lech *et al.*, 1984; Segal *et al.*, 1982). The effect of certain sugars and their complementary lectins on the adherence of 5 strains of *C. albicans* to BEC and VEC was studied by Critchley & Douglas (1987b). The results obtained were similar for both BEC and VEC. Four yeast strains, including GDH2346, displayed similar results, i.e., adherence was inhibited by L-fucose and winged pea lectin. This was not the case for strain GDH2023 which displayed decreased adherence for N-Ac-D-glucosamine and wheat germ agglutinin (Table VI). The EP from strains GDH2023 and GDH2346 was tested for its ability to bind immobilised sugars. Considerable proportions of "lectin-like proteins" which were specific for L-fucose, D-mannose and N-Ac-D-glucosamine occurred, but the profile differed for the 2 strains. From this

TABLE VI. Effect of principal inhibitory sugars and lectins on adhesion of *C. albicans* GDH2346 and GDH2023 to vaginal epithelial cells (Critchley & Douglas, 1987b)

STRAIN	SUGAR OR LECTIN	MEAN NO. (± SE) OF ADHERENT YEASTS PER 100 EPITHELIAL CELLS	RELATIVE ADHESION*	P+
GDH2346	L-Fucose	672 ± 41	70	<0.001
	N-Acetyl-D-glucosamine	951 ± 48	99	NS
	Winged-pea lectin	701 ± 28	73	<0.001
	Wheat-germ agglutinin	839 ± 59	87	NS
	None (PBS control)	959 ± 24	100	-
GDH2023	L-Fucose	1082 ± 13	98	NS
	N-Acetyl-D-glucosamine	615 ± 33	56	<0.001
	Winged-pea lectin	1024 ± 45	93	NS
	Wheat-germ agglutinin	756 ± 37	69	<0.001
	None (PBS control)	1101 ± 16	100	-

***Adhesion is expressed as a percentage of that in control mixtures for each strain**

+Probability values comparing adhesion in the presence of lectin or sugar with that in control mixtures

NS - Not significant

study it was proposed that *C. albicans* strain GDH2346 adheres to L-fucose-containing host receptors while GDH2023 would bind to N-Ac-D-glucosamine-containing receptors, respectively.

Fibronectin (Fn), a major surface glycoprotein of mammalian cells, was implicated in mediating adherence of *C. albicans* to epithelium (Skerl *et al.*, 1984). The adherence of yeast cells, pretreated with Fn, to VEC or BEC was decreased by 40 or 50%, respectively when compared to non-treated yeasts. The pathogenic bacterium *Staphylococcus aureus*, produced a protein which bound to the amino terminus of Fn (Espersen & Clemmensen 1982). Although *Streptococcus pyogenes* also bound to the amino terminus of Fn, the binding was at a different site from that of *Staphylococcus aureus* and mediated by lipoteichoic acid (Finlay & Falkow, 1989). Similarly, *Treponema pallidum* also bound to Fn but at a different site (Thomas *et al.*, 1985), which is the region to which integrins attach. Integrins are eucaryotic surface proteins involved in a variety of cellular functions including the binding of extracellular matrix and plasma proteins. Finlay & Falkow (1989) suggested that the filamentous haemagglutinin of *Bordetella pertussis* functions as the adhesin which binds to an integrin molecule, since the haemagglutinin included a conserved four-amino acid sequence (RGD) which partly shared homology with the RGD site of Fn. There is relatively greater information on

the nature of host receptors for pathogenic bacteria compared to fungi. The fact that *C. albicans* is capable of adhering to and colonizing a variety of tissue and host species indicates that a common cell surface molecule functions as the receptor, rather than that a specific receptor is present on any specific cell type or host.

C) METHODS FOR QUANTIFICATION OF ADHESION

To date, most of the experiments quantifying the adherence of *C. albicans* to epithelial cells involved a visual count, i.e., utilizing a light microscope (Ghannoum & Elteen, 1987; Kalo & Segal, 1988; Sandin *et al.*, 1987). A few researchers employed radiometric assays (Kimura & Pearsall, 1978; Reinhart *et al.*, 1985) or particle size analysis using a Coulter counter (Gorman *et al.*, 1986). Most of the studies involved the use of exfoliated donor epithelial cells. Standardized suspensions of *C. albicans* and epithelial cells were interacted in equal proportions. Apart from this, assay parameters varied considerably from study to study. This included differences in yeast culture conditions, type of host cells, ratio of yeasts to host cells, the assay medium and conditions of incubation.

Generally *C. albicans* yeasts were grown to the stationary phase in any one of a variety of media containing glucose ranging from 0,1 (Rotrosen *et al.*, 1985) to 10% (Cox, 1983). *C. albicans* was grown mainly to the stationary

yeast growth phase, i.e., for 18 h (Gorman *et al.*, 1986) to 24 h (King *et al.*, 1980). However, yeasts grown for 48 h were used by Centeno *et al.* (1983), while Sandin *et al.* (1987) considered yeasts grown for 13 to 15 h to be in the stationary phase of growth. The latter was not in keeping with Segal *et al.* (1982) who regarded yeast cells to be in the logarithmic stage of growth up to 18 h of culture. The growth temperature was either 25C (King *et al.*, 1980) or 37C (Kimura & Pearsall, 1978) depending on the growth medium used. The host cells were mostly exfoliated BEC and to a lesser extent VEC and uroepithelial cells (Sandin *et al.*, 1987). Corneocytes (Ray *et al.*, 1984) or cultured human cells (Rotrosen *et al.*, 1985; Sobel *et al.*, 1982) were also tested. The ratio of yeast to epithelial cells varied from 5:1 (Sandin *et al.*, 1982) to 1000:1 (King *et al.*, 1980). The most common assay medium was 0,1 M PBS ranging from pH 7,0 (Kimura & Pearsall, 1978) to pH 7,4 (Cox, 1983). Tissue culture medium was used by Ray *et al.* (1984) and Sobel *et al.* (1982). The interacted material was incubated at 37C with two exceptions in which incubation was carried out at room temperature (Collins-Lech *et al.*, 1984; Cox, 1983). The material was either not shaken (Collins-Lech *et al.*, 1984), gently shaken (Centeno *et al.*, 1983) or agitated up to a speed of 200 rpm (King *et al.*, 1980). The incubation time varied from 30 min (Cox, 1983) to 3 h (Kimura & Pearsall, 1978). Cells that were viewed microscopically were mostly air dried, fixed and stained. The number of epithelial

cells counted ranged from 30 (Centeno *et al.*, 1983) to 200 (Sandin *et al.*, 1982). While most assays involved the use of non-germinated viable yeast cells, the latter authors utilized killed cells with germ tubes. The adhesion mechanisms of *C. albicans* were investigated by the use of blocking agents in the adhesion assay. Isolated adhesin (Critchley & Douglas, 1987a; McCourtie & Douglas, 1985b), adhesin (Lee & King, 1983; Ray *et al.*, 1984; Segal *et al.*, 1982) or receptor analogues (Centeno *et al.*, 1983; Collins-Lech *et al.*, 1984; Critchley & Douglas, 1987b; Sandin *et al.*, 1982; Segal *et al.*, 1982) were tested resulting in competitive inhibition of adhesion. Alternatively, lectins were used to create steric interference with adhesion (Sandin *et al.*, 1982).

1. Visual

(a) Direct microscopic count

(i) Exfoliated epithelial cells

Yeast and host cells were interacted and the suspension viewed microscopically (Collins-Lech *et al.*, 1984; King *et al.*, 1980). Alternatively, the interacted material was filtered to remove non-adherent yeasts, as described by Kimura & Pearsall (1978). These authors devised a method which was widely used, with minor assay parameter modifications (Centeno *et al.*, 1983; Kalo & Segal, 1988; Ray *et al.*, 1984; Sandin *et al.*, 1987). Some of these modifications are reflected below. Polycarbonate 12 µm pore size membrane filters retained epithelial cells and

adherent yeasts. The cells were washed with 140 times the interacted volume, although Centeno *et al.* (1983) found that a 10 times washing volume was sufficient to remove non-adherent yeasts. The material was viewed directly on the transparent filters. Ghannoum & Elteen (1987) used a 20 µm pore size membrane filter to remove non-adherent yeasts and washed the retained cells with 2,5 times the interacted volumes. Epithelial cells and adherent yeasts were resuspended and viewed microscopically. An 8 µm pore size polypropylene membrane filter, in addition to differential centrifugation at 800 rpm to remove non-adherent *C. albicans*, was utilized by Sobel *et al.* (1982). The filter was pressed against a glass slide coated with a thin layer of albumin to which adherent yeasts and epithelial cells were transferred and viewed microscopically. Cox (1983) counted initial yeast cells and BEC using a Coulter counter. Standardized cell suspensions were interacted. The interacted suspension was centrifuged at 10x g to remove non-adherent yeasts. The pellet was viewed microscopically.

(ii) Cultured epithelium

Samaranayake & MacFarlane (1981) interacted *C. albicans* with HeLa cells grown on coverslips. The number of adhering yeasts were counted per mm^2 using an ocular graticule and a sampling stage on a light microscope. The same method was employed by Kalo & Segal (1988) but at different yeast culture conditions. Multilayered cultured

human vaginal epithelium and cultured human umbilical endothelium was interacted with *C. albicans* yeasts *in situ* for 2 h (Sobel *et al.*, 1982) and 24 h (Rotrosen *et al.*, 1985), respectively. The adherent yeasts were counted per field.

(b) Colony Count

C. albicans yeasts were interacted with host cultured endothelium (Rotrosen *et al.*, 1985). After washing with PBS, molten brain heart infusion agar was poured over the monolayer surface and allowed to solidify. Colonies, representing adherent *C. albicans*, were counted after incubation at 37C for 24 h.

2. Radiometric Assay

Interacted labelled yeasts and exfoliated epithelial cells were interacted and the suspension filtered through a 10 µm pore size polypropylene membrane filter which was washed 5 times with PBS and placed in solubilizer. After the addition of a scintillation cocktail, the counts of radioactive emissions were determined. A control of labelled yeast only was assayed and subtracted to obtain a true count of adherent yeasts (King *et al.*, 1980). Non-adherent yeasts were removed from the interacted cell suspension by centrifugation at 50x g. After protein digestion, counts were determined (Reinhart *et al.*, 1985).

3. Particle Count

C. albicans and BEC cell suspensions, which were standardized after a haemacytometer count, were interacted. Controls of either yeasts or BEC were subjected to the same incubation conditions as the interacted components. A Coulter counter, with a 100 μm orifice tube, was used to determine particle size. The count obtained for the interacted mixture was adjusted by subtracting the counts for the BEC control. The numbers of adherent yeasts were determined by subtracting the adjusted count from the count of yeast control (Gorman *et al.*, 1986).

II. MATERIALS AND METHODS

A) FUNGAL CULTURE

A clinical isolate of *C. albicans* was obtained from King Edward VIII Hospital, Durban. The fungus was maintained and subcultured every three months on glucose-yeast extract peptone agar (Table VII) and stored at 4C.

TABLE VII. Glucose-yeast extract peptone medium composition

INGREDIENT	$g.l^{-1}$
Glucose	20
Yeast Extract	10
Peptone	20
Agar	15
Water	1 000 ml

The yeast form of *C. albicans* was obtained by inoculating a loopful of the stock culture into 250 ml flasks containing 50 ml synthetic medium, the composition of which is shown in Table VIII. The culture was incubated overnight at 26C in a Braun Certomat shaker with incubator hood, at 150 rpm.

TABLE VIII. Amino acid synthetic medium for *C. albicans* (Lee *et al.*, 1975)

CHEMICALS	$g.l^{-1}$
SALTS:	
$(NH_4)_2SO_4$	5,0
$MgSO_4.7H_2O$	0,2
K_2HPO_4 (anhydrous)	2,5
NaCl	5,0
L-AMINO ACIDS	
Alanine	0,5
Leucine	1,3
Lysine	1,0
Methionine	0,1
Ornithine	0,0714
Phenylalanine	0,5
Proline	0,5
Threonine	0,5
Glucose	12,5
Biotin	0,001

The biotin was dissolved in 10 ml of distilled water and filter sterilized. Glucose and K_2HPO_4 (anhydrous) were each dissolved in 10 ml of distilled water and autoclaved at 110C at 10 lbs for 20 min. The rest of the components were dissolved in 970 ml distilled water and autoclaved at 110C at 10 lbs for 20 min. After autoclaving, the solutions were cooled and mixed. The pH was adjusted to 6,8 ± 0,05. The medium was stored at 4C.

B) TISSUE CULTURE

1. Preparation of Material

The following materials, unless stated otherwise, were prepared as follows for tissue culture:

(a) Phosphate buffered saline (PBS)

A 10x stock solution of PBS without calcium and magnesium (Gibco, U.K.) was diluted with distilled water to obtain a 0,15 M solution which was adjusted to pH 7,2 and autoclaved. The solution was stored at 4C.

(b) PBS with antibiotic-antimycotic solution

PBS was supplemented with 100 times antibiotic-antimycotic solution (Gibco) to effect a final concentration of 400 $U.ml^{-1}$ penicillin, 1 $mcg.ml^{-1}$ amphotericin B and 400 $U.ml^{-1}$ streptomycin.

(c) Medium with antibiotic-antimycotic solution (AA medium)

William's medium E (Gibco) was supplemented with penicillin and streptomycin (200 $U.ml^{-1}$ each) and 0,5 $mcg.ml^{-1}$ amphotericin B.

(d) Complete growth medium for tissue culture

The complete medium comprised William's medium E (without L-glutamine) with antibiotic-antimycotic solution, 20% foetal bovine serum (Whittaker M.A. Bioproducts, Inc.) and 2% low protein serum replacement (LPSR) -1 (Sigma, USA).

(e) Trypsinization medium

0,5% trypsin (1:250) and 0,2% EDTA in modified Puck's saline A (STV) (Gibco) was mixed with the AA medium in 1:1 ratio.

(f) Collagenase in medium

Collagenase from *Clostridium histolyticum* type 1A (Sigma) was dissolved in growth medium to obtain a concentration of 1 $mg.ml^{-1}$.

- Glassware for tissue culture purposes was treated with Super Decon (LASEC) and rinsed thoroughly with distilled H_2O.

- Wherever possible, sterile disposable pipettes and centrifuge tubes (Sterilin) were used for tissue culture and for particle size analysis.

2. Primary Vaginal Epithelium

Adult Wistar rats were sacrificed to obtain vaginal tissue. The organ was placed in cold PBS with antibiotic -antimycotic mixture. Connective tissue, blood vessels and fat were removed and the organ washed twice in cold AA medium supplemented with 0,01 ml anti-PPLO.ml^{-1} medium (Gibco). Thereafter various enzymic methods were attempted to disaggregate vaginal epithelium and on this basis, the tissue culture method may be divided into three groups, viz.:

- cold trypsinization or the use of collagenase (Table IX);
- trypsinization and the use of collagenase (Table X); and
- cold trypsinization and the use of collagenase (Table XI).

For trypsinization purposes, the organ was placed in the trypsinization medium and incubated at 37C. The method for trypsinization in the cold was modified from Paul (1975). The organ in trypsinization medium was incubated at 4C overnight. This was followed by incubation at 37C.

In both instances the organ was washed 2x with complete growth medium.

TABLE IX. Cold trypsinization or the use of collagenase

ENZYME	OVERNIGHT INCUBATION AT 4C	INCUBATION TIME AT 37C	HORMONAL STATE
Trypsin	+	1 h	U*
Collagenase	+	5 h	U
"	+	8 h	U
"	-	4 h	U
"	-	Overnight	U; O

U* = Unknown

O = Oestrus

D = Dioestrus

M = Metoestrus

P = Proestrus

Regardless of the enzymatic digestion method used, after the first incubation at 37C, the tissue was placed in a sterile plastic petri dish lid and cut into small pieces

approx. 2-3mm in size (Owens, 1976), using a no. 22 scalpel. Tissue fragments were placed into collagenase in medium and incubated at 37C, unless indicated otherwise.

TABLE X. Trypsinization and the use of collagenase

TRYPSIN INCUBATION TIME AT 37C	COLLAGENASE INCUBATION TIME AT 37C	HORMONAL STATE-
1 h	3 h	O*
1 h	4 h	O; P
1 h	5 h	O; P; U
1 h	6 h	D; U
2 h	5 h	U

*See Table IX

The tissue was centrifuged at 150x g for 5 min and the pellet washed with AA medium. The pellet was resuspended in either foetal bovine serum or AA medium and the tissue pipetted into 25 cm^2 tissue culture flasks (Sterilin or Nunclon). The tissue fragments were distributed evenly in the flasks which were incubated (with caps loosened) in a CO_2 incubator (Precision Scientific, USA) in an atmosphere

of 5-7% CO_2 overnight or for 45 min to 1 h (R. Haniff, University of Natal, personal communication). In the latter instance medium was removed from the flask prior to incubation. Thereafter, 2 ml of complete growth medium was added carefully along the side of each flask. The flask was tilted slowly, allowing the medium to bathe the tissue. Similarly, 2 ml growth medium (without serum) was added to each flask incubated overnight. Flasks were left undisturbed in the CO_2 incubator for 7 d or until the medium acidified, as indicated by a change of the phenol red indicator in the medium to yellow. Cultures were maintained by changing the acidified medium whenever necessary, which was usually every 2 to 3 d. Fibroblast growth was controlled by trypsinization with STV on a weekly basis, after the culture was 10 d old. The trypsinization procedure was modified from Paul (1975). One ml of STV was added to the culture which was incubated for 2 min at 37C. The STV was removed, the cap of the flask tightened and the flask incubated at 37C for a further 2-3 min. The action of trypsin was stopped by adding 2 ml William's medium containing foetal bovine serum. The tissue was washed twice with William's medium and fed 4 ml of complete growth medium. The hormonal status of the rats was determined prior to dissection, wherever indicated, by screening vaginal scrapings to observe epithelial cell types. A chi-squared test for tissue trypsinized in the cold and treated with collagenase for 4-5 h, was carried out to determine

whether the hormonal state of the animal made any difference to growth of tissue (Lee & Lee, 1982).

TABLE XI: Cold trypsinization and the use of collagenase

TRYPSIN INCUBATION TIME AT 37C	COLLAGENASE INCUBATION TIME AT 37C	HORMONAL STATE
15 min	2 h	O*
15 min	5 h	U
15 min	8 h	U
1 h	1 h	O
1 h	2 h	O; P; M
1 h	3 h	O; P
1 h	4 h	U; O
1 h	5 h	U; P
1 h	6 h	U**
1 h	7 h	U
1 h	8 h	U
1 h	Overnight	U

*See Table IX. **Including overnight + 24 h cold trypsinization

Cultures were viewed using an inverted microscope (Zeiss West Germany)) fitted with a camera (Zeiss Ikon). Low magnification photographs of the cultures were taken on a photomicroscope (Wild M400).

3. Buccal Epithelial Cell Line

A buccal epithelial cell line was donated by Mr I.E. Karodia. The cultures were maintained in an atmosphere of 5-7% CO_2 in the CO_2 incubator and the medium changed weekly. The medium comprised modified Eagle's Minimum Essential Medium with Earle's salts supplemented with 10% foetal bovine serum, 2% LPSR, 400 $U.ml^{-1}$ penicillin and streptomycin, 0,5 $mcg.ml^{-1}$ amphotericin B, 0,01 ml anti-$PPLO.ml^{-1}$ medium and 0,01 ml non-essential amino $acids.ml^{-1}$ medium.

C) INTERACTION OF YEAST AND EPITHELIAL CELLS

1. Quantification

(a) Harvesting of cells

(i) Yeast cells (YC)

C. albicans YC were harvested by centrifuging 5 ml of the culture in a Sterilin centrifuge tube at 200x g for 5 min. The pellet was washed and resuspended in sterile PBS. The YC concentration was adjusted to 10^5 $cells.ml^{-1}$ after counting in a haemacytometer.

(ii) Vaginal epithelium

All solutions and media for dissociating the tissue were prewarmed to 37C. A minimum of 24 h prior to interaction, cultures were trypsinized using STV for 2 min at 37C. Tissue cultures were washed with PBS. Dissociation medium (Sigma) was added to each culture and the flasks incubated at 37C for 5 min. The dissociation medium was removed and 5 ml of William's medium added to each flask. A "policeman" was used to gently release adherent tissue from the flask. The medium was drawn up into a syringe fitted in a 20G needle to break up clumps of cells. The needle was replaced in a 25 mm Sartorius filter holder containing 100 T Nytex of 58 µm mesh size (donated by Bishop Merchandising CC, Durban), which was sterilized in 70% alcohol overnight and air dried in a laminar airflow cabinet (Labotec). Cells of < 58 µm were collected in a Sterilin centrifuge tube and centrifuged at 150x g for 5 min. Cells grown from the same animal were pooled. The pellet was washed and resuspended in sterile PBS to 10^2 cells.ml^{-1} after counting in a haemacytometer. Epithelial cells were harvested first and held at 4C, until immediately prior to interaction.

An adult Wistar rat was sacrificed and the vagina removed. Connective tissue, blood vessels and fat were removed and the organ washed twice in cold William's medium with antibiotic-antimycotic mixture. The organ was placed in a Sterilin centrifuge tube containing William's medium and

collagenase (1 $mg.ml^{-1}$) and incubated overnight (18 h) in the CO_2 incubator. The tissue was centrifuged at 150x g for 5 min and washed with William's medium. The tissue was syringed several times through a 20G needle to break up clumps, subjected to filtration and treated as described above for cultured vaginal epithelium.

(iii) Buccal epithelial cell line

Buccal epithelial cell line cultures which were washed with PBS were each treated with 1 ml dissociation medium and incubated at 37C for 10 min. A volume of 4 ml William's medium was added to each flask. The cell suspension was subjected to filtration and treated as described for vaginal epithelial cells.

(iv) *Escherichia coli*

An overnight culture of *E. coli* strain B grown on nutrient agar at 37C was suspended in sterile PBS. Cells were counted in a haemacytometer and the cell concentration was adjusted to 10^6 - 10^8 $cells.ml^{-1}$.

(b) Interaction

Yeast and epithelial cells were interacted in sterile stoppered glass test tubes and incubated at 37C for 90 min. The control comprised PBS added to YC (Table XII).

The effect of mannose or conA on the interaction was determined by adding either 100 $mg.ml^{-1}$ α-methyl mannoside or 250 $\mu g.ml^{-1}$ conA (Sigma) to the interacted cell suspension and to the control prior to incubation. To determine the effect of bacteria on the interaction, a suspension of *E. coli* was added to the interacted cell suspension and to the control.

TABLE XII. Interaction of yeast and epithelial cells

	VEC/BEC	YC	PBS
Control	-	0,5 ml	0,5 ml
Test	0,5 ml	0,5 ml	-

(c) Microscopic count

A suspension of interacted material was stained with crystal violet and 3 samples were viewed using a Zeiss microscope. A total of 100 VEC were counted and the number of adherent VEC was determined. VEC with 20 or more YC were regarded as adherent. The total number of YC adhering to 100 cells counted at random, was determined.

(d) Particle size analysis

The interacted cell suspension was filtered through a 12 µm (Schleicher and Schuell) membrane filter and washed in 20 times its volume. Five ml of the filtrate was collected in a Sterilin centrifuge tube and centrifuged at 200x g for 5 min. Samples of the uninteracted epithelial cells and YC which were held at 4C, were also centrifuged at 150x and 200x g, respectively.

The cells were fixed by resuspending each pellet in 10% formaldehyde for 30 min at 4C. The cells were washed 3 times with PBS that was filtered through a 0,2 µm disposable filter (Millipore). The interacted material, control (C) and the initial epithelial cell suspension were each resuspended in 2 ml filtered PBS and transferred to Sterilin containers. The initial yeast suspension (I) was resuspended in 5 ml filtered PBS and 0,1 ml was added to 4,9 ml filtered PBS in a Sterilin container. The material was stored at 4C until analyzed.

A Criterion light blockage Particle Size Analyzer model PC-320 (HIAC Division, Pacific Scientific Co.) linked with a CMB 60 sensor (range 1 to 60 µm) in an automatic bottle sampler, was used to count the number of cells. The flow rate was regulated to 8 $s.ml^{-1}$ and the samples diluted so that the number of particles was within the concentration limit specified for the CMB 60 sensor. The particles were analyzed per unit volume on the Delta mode where each

channel counted particles between two adjacent thresholds. The thresholds were set to count particles as follows: 2 µm, 3 µm, 5,6 µm, 16 µm, 35 µm, 50 µm.

The YC were counted between 2 and 5,6 µm and epithelial cells between 16 and 50 µm, thus the readings for 2 and 16 µm were rejected. Three samples of 0,5 ml each were read per suspension and the average taken. Control samples of filtered PBS were taken into consideration. The particle size counts of initial cell suspensions were used in calculating adherence values as opposed to the counts obtained using the haemacytometer. Statistical analysis was carried out by Student's *t*-test to evaluate the effect of mannose, conA and *E. coli* on adherence (Lee & Lee, 1982).

2. Electron Microscopy

(a) *In situ*

C. albicans was interacted with cultured vaginal epithelium *in situ* for 1 h at 37C. The tissue was washed to remove non-adherent YC. The material was fixed with 1% glutaraldehyde in PBS containing 0,002% ruthenium red solution for 2 h at room temperature. The glutaraldehyde was removed and the material washed 3 times with PBS containing 0,002% ruthenium red solution. At this stage the area to be investigated was cut out using a heated blade. For SEM, the interacted tissue and the underlying plastic was cut in a circle the size of a SEM brass stub.

For TEM purposes, the material was cut into smaller pieces approximately 3 x 4 mm. The plastic pieces with material were postfixed in microfuge tubes, to which 1% aqueous OsO_4 in PBS with 0,002% ruthenium red was added, for 1 h at room temperature. The material was washed with distilled water and stained with 2% uranyl acetate for 30 min. The material was dehydrated through a series of absolute alcohol (99,9%) dilutions for 15 min each: this comprised 50%, 70%, 95%, 100% and 2 x dry 100%. The material was left in dry 100% alcohol overnight. The specimens were treated either for SEM or TEM.

For SEM purposes, the specimen was critical point dried with CO_2 in a Polaron E3000 critical point dryer and attached to a brass stub using double sided tape. The specimens were coated with gold in a Polaron E5000 sputter coating unit, and viewed using a scanning electron microscope (Philips 500). For TEM purposes, the material was infiltrated with pure Spurr's resin for 6 h. The pieces were orientated in blocks such that sections would be cut perpendicular to the plane of the monolayer. Pure Spurr's resin was added to each block and the "coffin" incubated for 8 h at 70C for polymerisation.

Sections were cut on a Reichart OMU3 ultramicrotome using glass knives. Ultrathin sections were collected onto copper grids and post-stained for 6 min with lead citrate. Sections were viewed using a Philips 301 TEM.

(b) Dissociated Cells

Cultured VEC were dissociated and interacted with *C. albicans* YC. A drop of the interacted suspension was placed on a small coverslip which was processed for SEM, as described earlier.

III. RESULTS

A) TISSUE CULTURE

1. Growth and Maintenance

The results obtained using different methods to culture vaginal epithelium are presented in Table XIII and XIV. A single attempt of trypsinization on its own was unsuccessful. Although some success was achieved with overnight collagenase treatment on its own, very little growth was observed. The best results were obtained with a combination of trypsin and collagenase treatments for 1 h and 2 to 4 h, respectively. Trypsinization in the cold, which was done out of convenience, produced results that were comparable with the conventional trypsinization procedure of incubation at 37C. It was surprising that tissue trypsinized overnight and for a further 24 h in the cold, still resulted in good growth. Growth was obtained with the collagenase incubation time ranging from 2 h to 8 h, but not overnight. It should be noted that the hormonal state of the donor animal was of significance in determining successful growth. The probability of the hormonal state affecting growth was 70 to 80% as determined by chi-squared tests for tissue trypsinized in the cold and treated with collagenase for 4-5 h. Numerous attempts using tissue from rats in proestrus or oestrus were mostly successful. A positive result was also obtained for tissue from rats in metoestrus, but not dioestrus. Tissue which was incubated for 1 h

TABLE XIII. Results of vaginal epithelium tissue culture using either trypsin or collagenase treatments

HORMONAL STATE	ENZYME 1	OVERNIGHT INCUBATION TEMPERATURE (C)	INCUBATION TIME AT 37C	ENZYME 2	INCUBATION TIME AT 37C	NO. OF SUCCESSES/NO. OF ATTEMPTS
*U	T	4	1 h	-	4 h	0/1
U	C	4	5-8 h	-	-	0/2
U	-	-	-	C	4 h	0/1
U	C	37	-	-	-	0/1
O	C	37	-	-	-	2/2

*C = collagenase; D = dioestrus; M = metoestrus; O = oestrus; P = proestrus; T = trypsin; U = unknown

at 37C in the CO_2 incubator without foetal bovine serum showed increased adherence to the culture flask compared to overnight incubation with foetal bovine serum.

Epithelial outgrowths were macroscopically evident after 5 to 7 d of incubation. The outgrowth of tissue is evident

TABLE XIV. Results of vaginal tissue culture using sequential trypsin and collagenase treatment

HORMONAL STATE	ENZYME 1	OVERNIGHT INCUBATION TEMPERATURE (C)	INCUBATION TIME AT 37C	ENZYME 2	INCUBATION TIME AT 37C	NO. OF SUCCESSES/NO. OF ATTEMPTS
*U	T	-	1 h	C	5-6 h	3/4
U	T	-	2 h	C	5 h	0/1
D	T	-	1 h	C	6 h	0/1
O	T	-	1 h	C	3-5 h	7/8
P	T	-	1 h	C	4-6 h	9/9
U	T	4	15 min	C	5-8 h	1/3
O	T	4	15 min	C	2 h	0/2
**U	T	4	1 h	C	4-8 h	9/16
U	T	4	1 h	C	overnight	0/1
O	T	4	1 h	C	1 h	0/1
O	T	4	1 h	C	2-4 h	19/20
M	T	4	1 h	C	2 h	1/1
P	T	4	1 h	C	2-5 h	6/9

*See Table XIII

**Including trypsinization in the cold - overnight + 24 h

in Fig. 9. Most of the growth, measured as an increase in growth diameter, took place up to the third week of

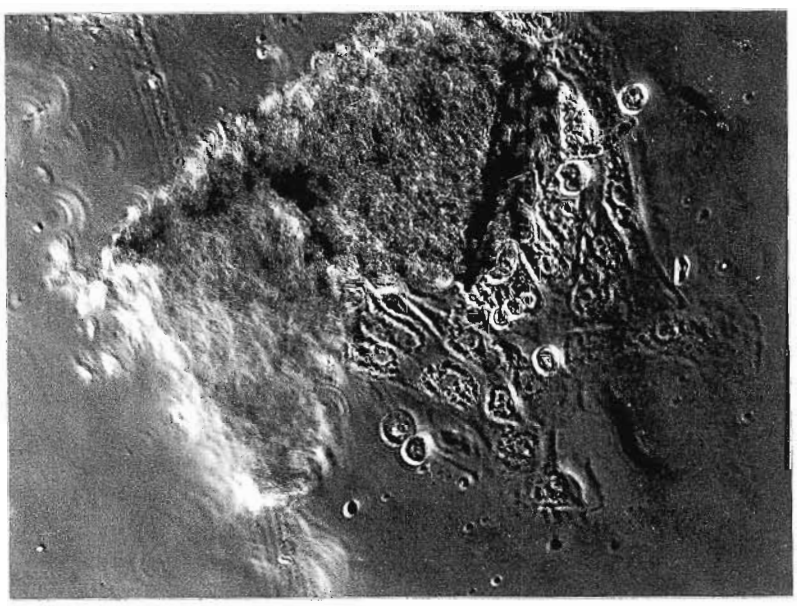

FIG. 9. Initial outgrowth of epithelial tissue (x160). Zeiss inverted microscope

incubation; with some outgrowths achieving a diameter of 20 mm at this stage. The tissue continued to grow and remained viable at 6 weeks. Growth was almost always hindered by fibroblasts which made their appearance at about 8 d of incubation. Fibroblasts, if left unchecked, tended to surround epithelial cell islands, physically restricting epithelial growth. The fibroblastic growth was successfully controlled by weekly trypsinization. This procedure removed most of the fibroblasts, while

leaving epithelial cell islands relatively undisturbed. Epithelial tissue is shown in Fig. 10 and the characteristic epithelial morphology in Figs. 11 and 12. The tissue showed some degree of multilayering after 3 weeks of culture as illustrated in Fig. 13.

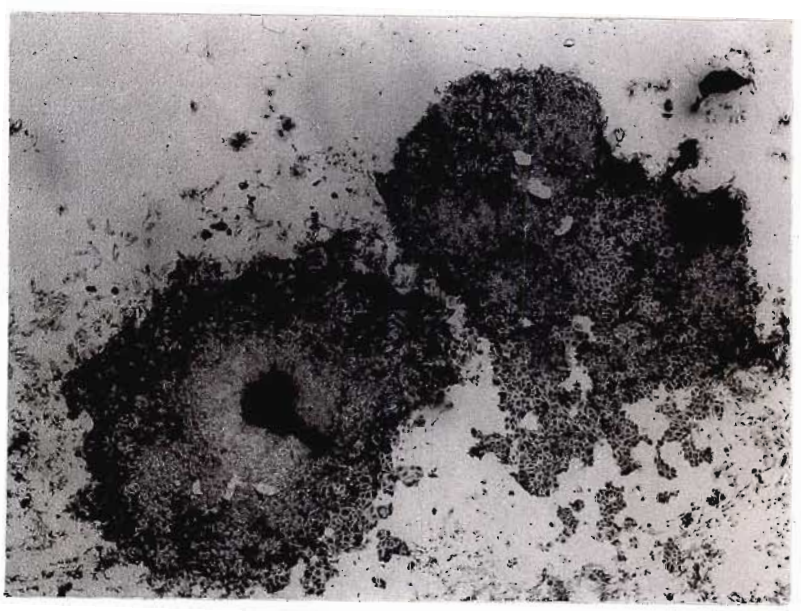

FIG. 10. Two epithelial outgrowths stained with crystal violet at 1 mth of culture (x1.8). Wild M400 photomicroscope

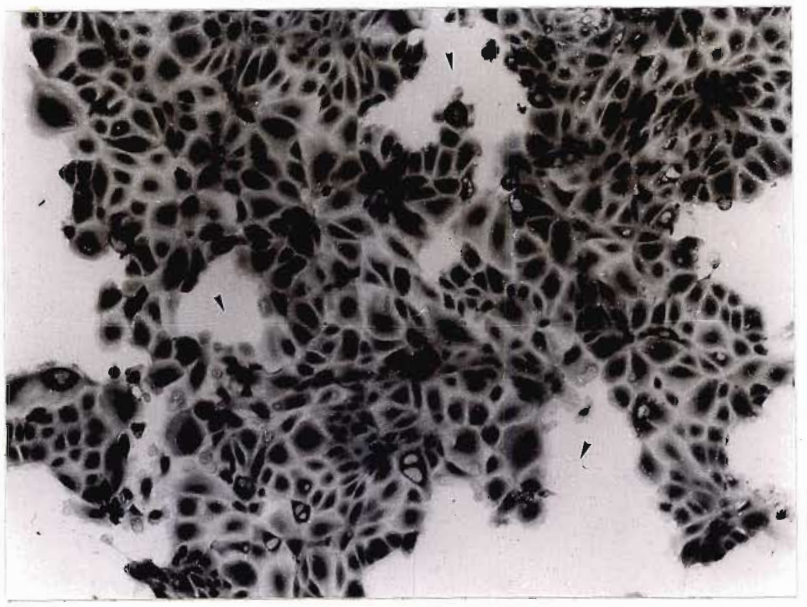

FIG. 11. Outer region of epithelial outgrowth stained with crystal violet. The gaps between cluster of cells (arrowheads) were caused by trypsinization for the removal of fibroblasts (x10.9). Wild M400 photomicroscope

FIG. 12. Epithelial tissue fixed with 1% glutaraldehyde showing characteristic epithelial morphology (x160). Zeiss inverted microscope

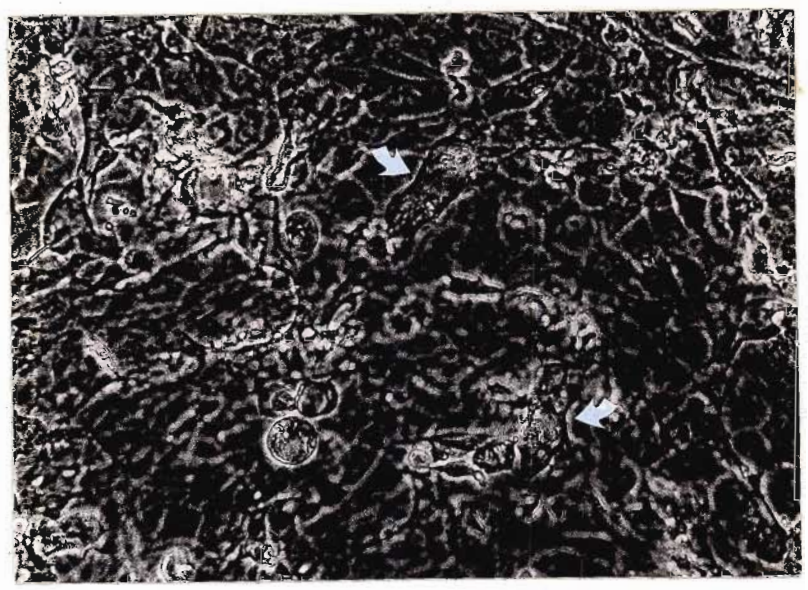

FIG. 13. Multilayer of cells indicated by arrows, observed at 28 d of epithelial tissue culture (x200) Zeiss inverted microscope

2. Dissociation of Epithelial Cells

The dissociation medium on its own was not sufficient to remove VEC from the tissue culture flask and it was necessary to use a 'policeman' to physically loosen adherent tissue. It was easier to dissociate BEC than the primary cultured cells and the use of a policeman was not required. However, a longer incubation period for dissociation was necessary. The cell clusters were further dissociated by aspirating the material a few times through a 20 G needle. The cells were filtered through 58 µm mesh size 100 T Nytex to remove any undissociated clumps of cells. This ensured that only cells that were approximately < 58 µm were collected for the interaction.

This procedure was necessary for the particle count as particles larger than 60 µm in diameter would result in sensor blockage. For the same reason, filtered PBS was used for final washings of the material.

B) ADHERENCE ASSAY

1. Particle Count

The YC particle count was obtained by summing the mean of the differences of the counts for the 3 and 5,6 µm thresholds. Similarly, epithelial cell counts were determined by summing the mean of the differences of the readings obtained for the 35 and 50 µm thresholds. These results were multiplied by the dilution factor. The total number of adherent YC was determined by applying the following equation:

Total number of adherent yeasts = control (C) count
- interacted YC count

The mean values of filtered PBS counts were negligible and were therefore ignored. The percentage adherence of YC was calculated as follows:

$$\text{Percentage adherent YC} = \frac{\text{Total number of adherent yeasts}}{\text{Initial YC count (I)}} \times 100$$

$$\frac{}{2}$$

The results of candidal adherence to VEC, BEC and non-cultured cells from the rat are described in Fig. 14.

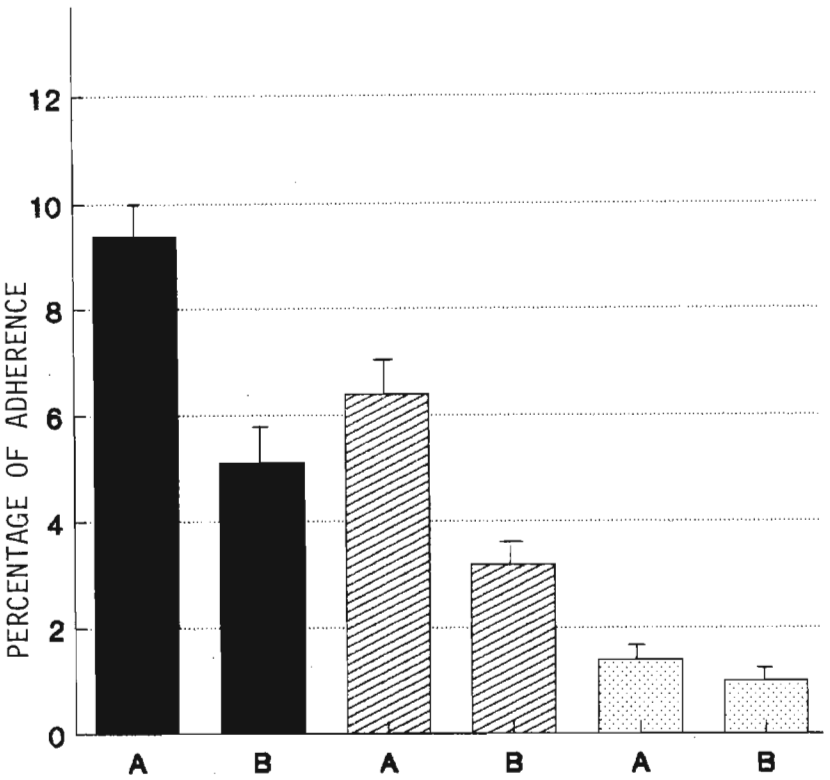

FIG. 14. The percentage adherence of YC to epithelial cells

A Cells counted by particle size analysis.

B Cells counted microscopically.

VEC.

BEC.

Non cultured cells from the rat.

Bar represents standard error of mean of 3 samples

The highest percentage adherence was obtained for VEC followed by BEC and non-cultured cells, respectively. The effect of mannose, con A and *E. coli* on the adherence of

C. albicans to VEC and BEC is shown in Table XV. In each case, adherence was significantly reduced when compared to the control with YC alone. The overall highest and lowest inhibition of adherence was for BEC treatment with conA and *E. coli* respectively. Treatment with mannose resulted in a greater reduction in adherence for VEC than BEC, while the opposite was true for treatment with conA. For both VEC and BEC, the addition of *E. coli* resulted in higher relative adherence compared to mannose or conA treatment.

2. Microscopic Count

The results obtained for the adherence of *C. albicans* to the different epithelial cell types, as described in Fig. 14, indicated that a lower count was obtained in each instance for the visual assay compared to the particle count. The least difference was observed for the lower values, i.e., the adherence of YC to non-cultured cells from the rat. The effect of mannose, conA and *E. coli* on the adherence of *C. albicans* to epithelial cells, as observed visually, is shown in Table XVI. The adherence was significantly inhibited in each case, with the greatest inhibition by conA for BEC and the least by *E. coli* for BEC. The percentage reduction in adherence by mannose and conA was 2% less than that obtained using the particle count method, with the exception of BEC treatment with mannose and VEC treatment with conA which was higher by 7% and the same, respectively. The results for *E. coli*

were much lower than that observed for the particle count, i.e. 11% and 24% lower for VEC and BEC, respectively.

TABLE XV. The inhibition of adherence of *C. albicans* to epithelial cells as determined by the particle count method

TREATMENT	NUMBER OF ADHERENT YEASTS PER 100 EPITHELIAL CELLS*	% ADHERENCE	PROBABILITY**
VEC			
Control	9374 ± 587	100	
Mannose	469 ± 112	5	< 0.001
ConA	750 ± 164	8	< 0.001
E. coli	1781 ± 57	19	< 0.001
BEC			
Control	6451 ± 578	100	
Mannose	1097 ± 145	17	< 0.001
ConA	193 ± 9	3	< 0.001
E. coli	2830 ± 656	44	< 0.01

* mean value ± standard error of mean of 3 samples

** probability relative to respective control, as determined by two-tailed t-test for v = 4 (Lee & Lee, 1982)

TABLE XVI The inhibition of adherence of *C. albicans* to epithelial cells as observed by visual assay

TREATMENT	NUMBER OF ADHERENT YEASTS PER 100 EPITHELIAL CELLS*	% ADHERENCE	PROBABILITY**
VEC			
Control	5064 ± 662	100	
Mannose	354 ± 44	7	< 0.001
ConA	384 ± 19	8	< 0.001
E. coli	411 ± 81	8	< 0.001
BEC			
Control	3214 ± 299	100	
Mannose	336 ± 52	10	< 0.001
ConA	159 ± 49	5	< 0.001
E. coli	643 ± 111	20	< 0.001

*; ** See Table XV

The adhesive capacity of epithelial cells, as determined by their ability to bind 20 or more YC, is described in Table XVII. A comparison of the epithelial cell types indicated that VEC were most adherent, followed by BEC and non-cultured cells from the rat. Treatment with mannose, conA and *E. coli*, resulted in 100% loss of adherent ability of VEC and BEC, with the exception of treatment with *E. coli* for BEC. In the latter instance the adhesive capacity of BEC was reduced by 94%.

TABLE XVII. The adhesive capacity of epithelial cells and the effect of inhibitory treatments

EPITHELIAL CELL	TREATMENT	ADHESIVE CAPACITY (%)*
Non-cultured VEC	-	35 ± 5
Cultured VEC	-	82 ± 15
"	Mannose	0 ± 0
"	ConA	0 ± 1
"	*E. coli*	0 ± 0
BEC	-	65 ± 8
"	Mannose	0 ± 1
"	ConA	0 ± 0
"	*E. coli*	4 ± 2

*Epithelial cells capable of binding 20 or more YC/cell

C) ELECTRON MICROSCOPY

1. Scanning Electron Microscopy (SEM)

The *in situ* SEM of the interacted YC and VEC revealed that *C. albicans* adherence was greater for exfoliating cells than cells that were still attached to the underlying plastic (Fig. 15). The adherence of *C. albicans* resulted in cavitation of the VEC at the area of contact (Figs. 16a

and b). Extracellular material was observed linking YC to the host cell (Fig. 16b). Germ tubes were produced away from the site of contact of the adherent yeast cell (Fig. 17). The adherence of *C. albicans* to dissociated VEC is shown in Fig. 18.

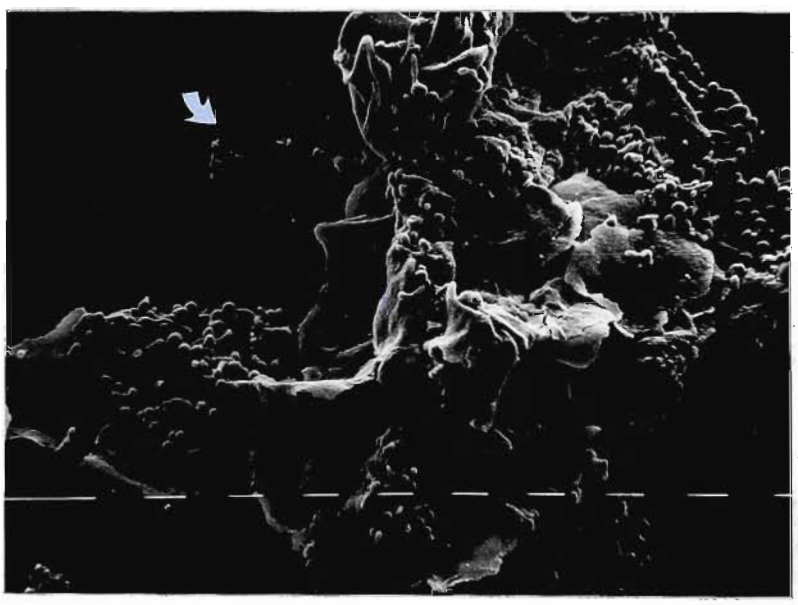

FIG. 15. Scanning electron micrograph of the adherence of *C. albicans* to cultured vaginal tissue *in situ*. Arrow indicates yeasts attached to epithelial cells adherent to plastic. Bar = 10 µm

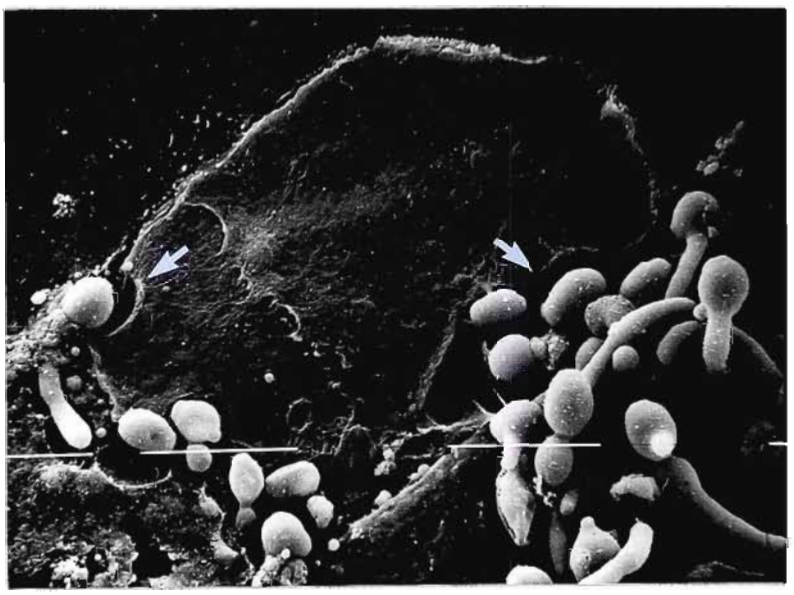

FIG. 16a. Cavity formation (arrows) by *C. albicans* YC adhering to a cultured VEC *in situ*. Bar = 10 µm

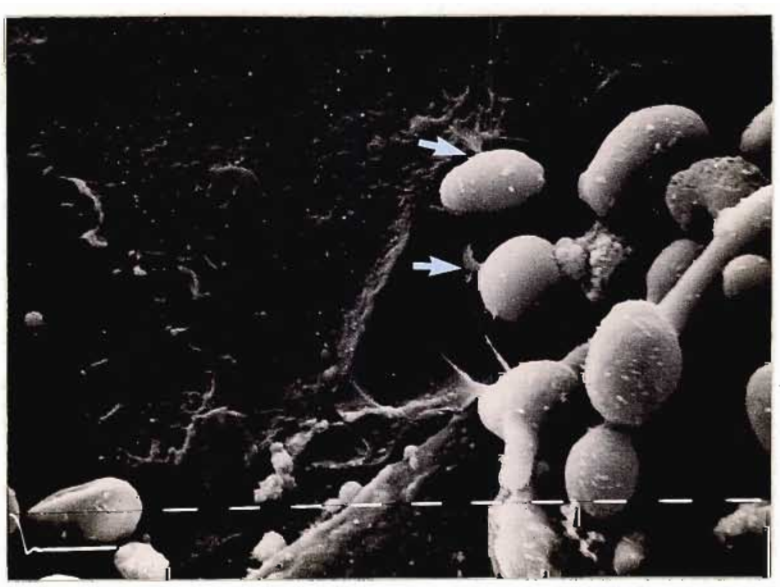

FIG. 16b. At higher magnification, extracellular material (arrows) in contact with the YC and epithelial cell surfaces. Bar = 1 µm.

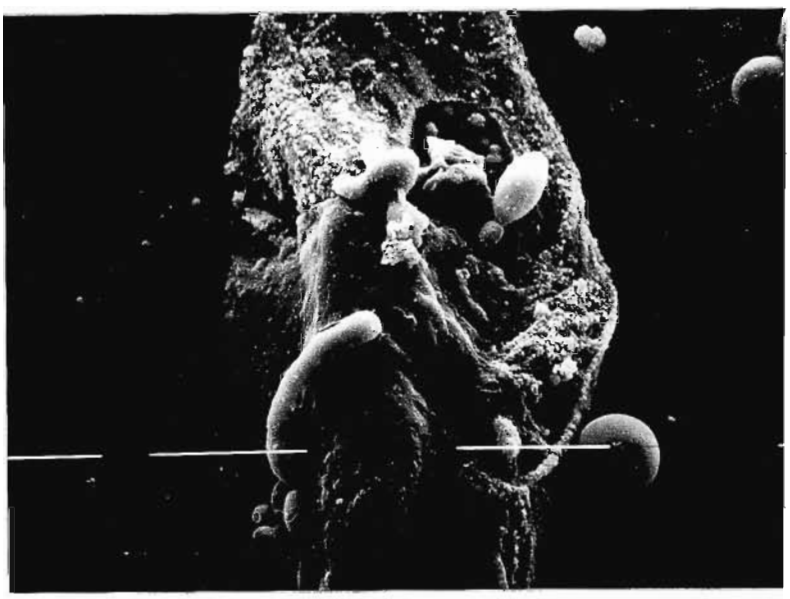

FIG. 17. Germ tube production by adherent *C. albicans* yeast on cultured VEC *in situ*. Bar = 10 µm

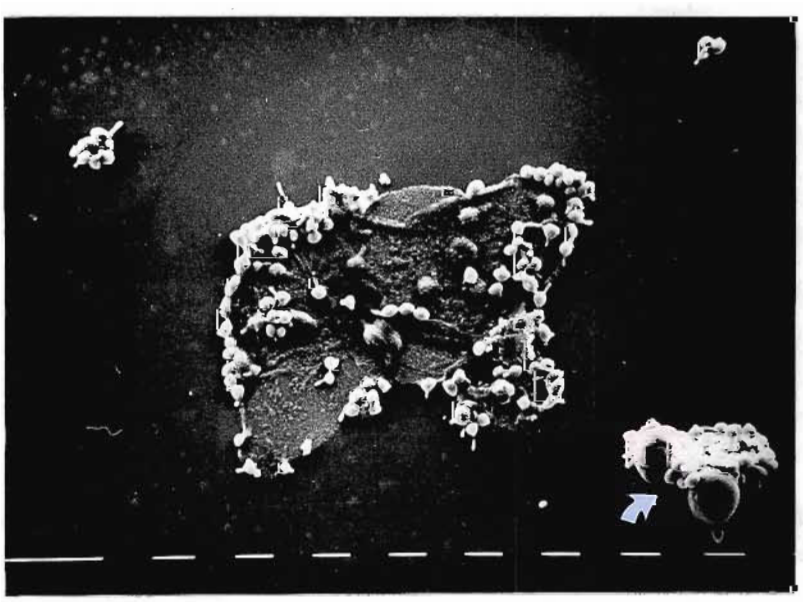

FIG. 18. The adherence of *C. albicans* to dissociated cultured VEC. Arrow indicates trypsinized cells. Bar = 10 µm

2. Transmission Electron Microscopy (TEM)

The ultrastructure of the adherence of *C. albicans* to VEC *in situ* is shown in Fig. 19. The YC were in close association with host tissue. In addition, the coadherence of YC to each other were also observed (Fig. 20). The adherence of YC to host tissue and each other was mediated by ruthenium red stained outer cell wall layer (Figs. 20 and 21). In Fig. 21, the ruthenium red-positive layer was closely associated with the host cell membrane, while in Fig. 22, distinct fibrils are evident along the outer layer of the yeast cell wall which was in close proximity to the host

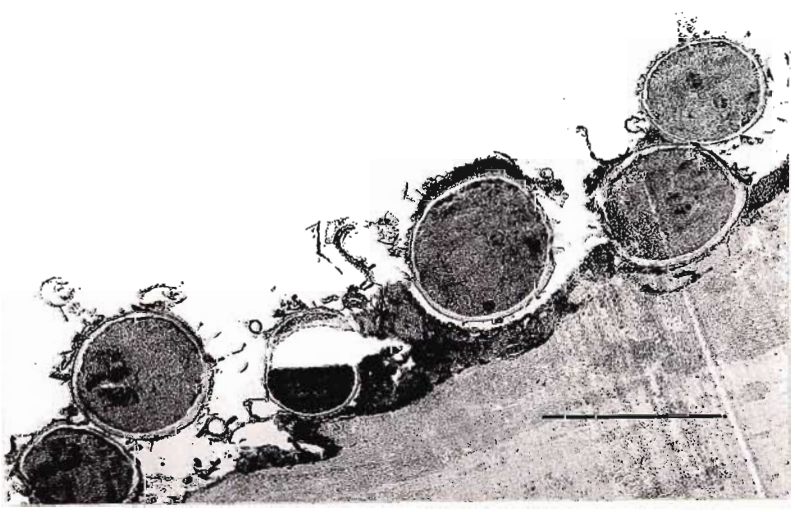

FIG. 19. Transmission electron micrograph of *C. albicans* YC adhering to cultured VEC *in situ*. Bar = 10 μm

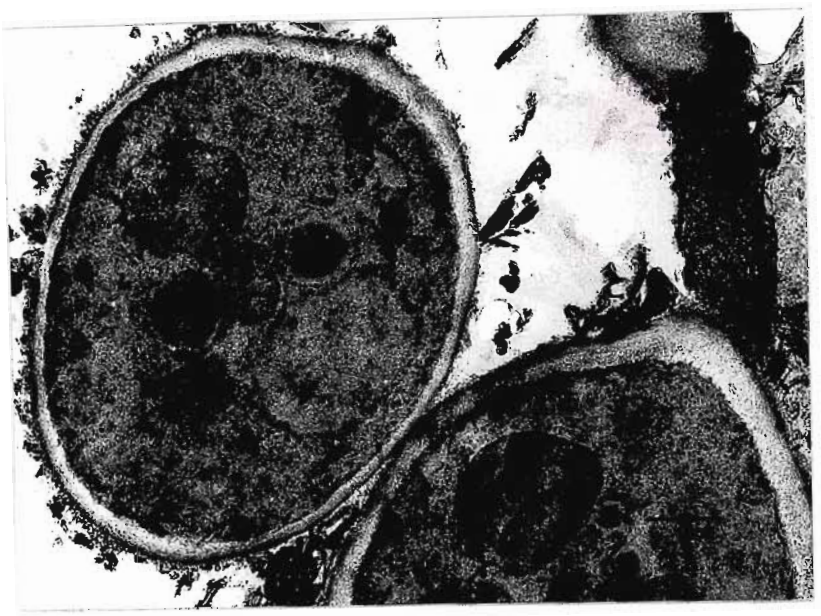

FIG. 20. Coadherence of *C. albicans* YC. Bar = 1 µm

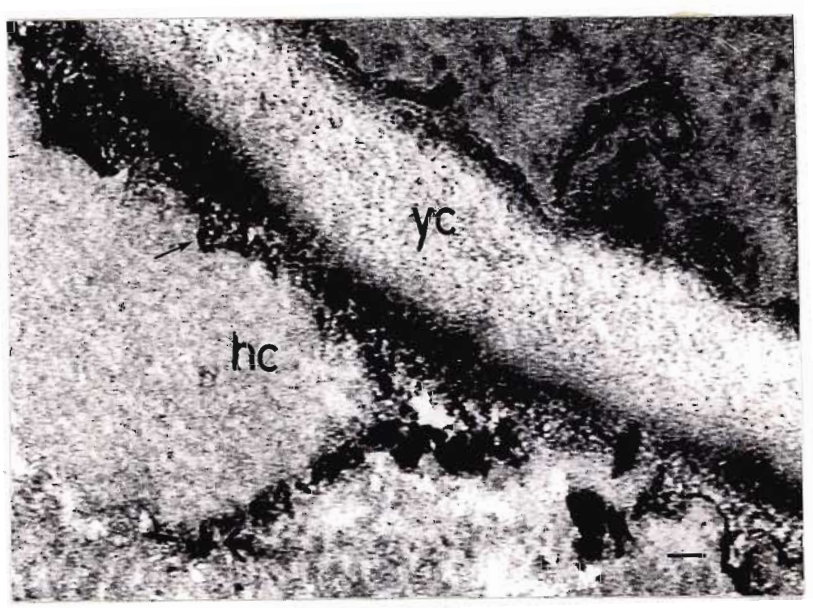

FIG. 21. *C. albicans* yeast cell wall and host cell membrane interface indicated by arrow. Bar = 100 nm. yc = yeast cell. hc = host cell

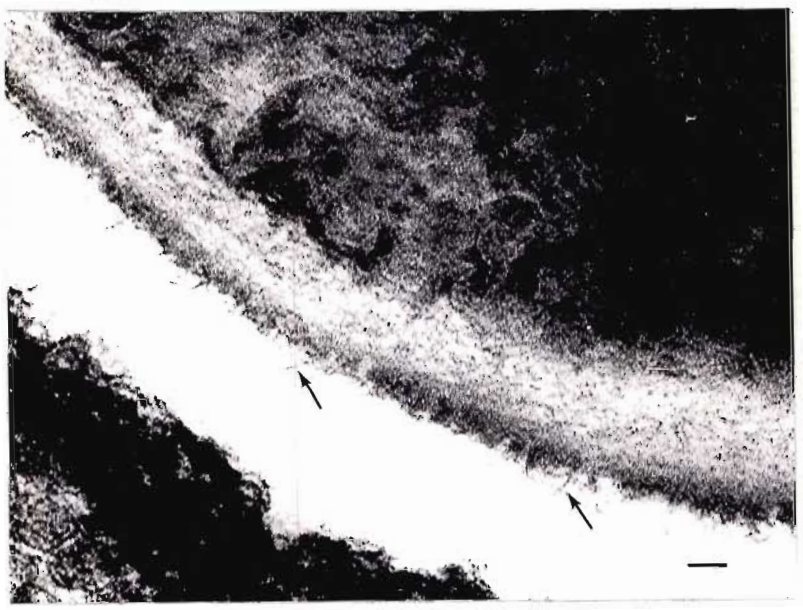

FIG. 22. Fibrillar outer wall layer of *C. albicans* indicated by arrows. Bar = 100 nm

tissue. The host cell surface was covered with large amounts of densely stained material at some points and this material was also found on the outer surface of the yeast (Fig. 19). At a higher magnification, this material was found to comprise, in part, membranous matter which was not organized into any definite structures (Fig. 23). Rough endoplasmic reticulum (RER) was well developed in the cultured host cells (Fig. 24).

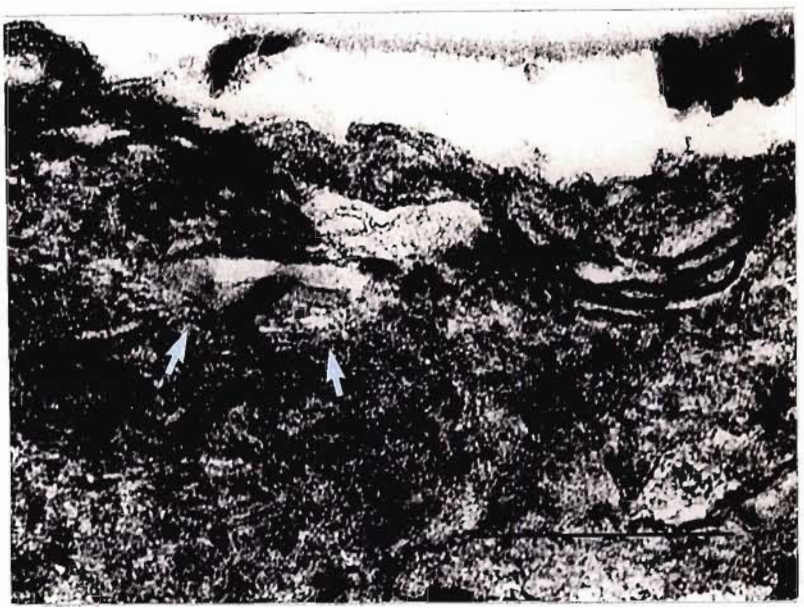

FIG. 23. Membraneous material (arrows) present on the part of the host cell surface. Bar = 1 μm

FIG. 24. Rough endoplasmic reticulum (arrows) in cultured VEC. Bar = 1 μm

IV. DISCUSSION

The *C. albicans* yeast and cultured host cell interaction was studied previously using host cell lines (Kalo & Segal, 1988; Makrides & MacFarlane, 1983; Samaranayake & MacFarlane, 1981), in one instance primary human vaginal epithelium (Sobel *et al.*, 1982) and in another endothelial epithelium (Rotrosen *et al.*, 1985). Although the latter authors observed the ultrastructure of *C. albicans* interacted with endothelium, Sobel *et al.* (1982) used primary vaginal epithelium to quantify adhesion *in situ* and did not make any ultrastructural observations of the interaction. In this study, the primary culture of normal rat vaginal tissue provided a host model and the author is unaware, to date, of any other ultrastructural studies of the interaction between cultured vaginal cells and *C. albicans*. Most of the studies on adhesion have concentrated on the use of exfoliated donor host cells (Centeno *et al.*, 1983; Collins-Lech *et al.*, 1984; Cox, 1983; Ghannoum & Elteen, 1987; Gorman *et al.*, 1986; Kimura & Pearsall, 1978; King *et al.*, 1980; Ray *et al.*, 1984; Sandin *et al.*, 1982; Segal *et al.*, 1984). Non-cultured tissue has been used for electron microscopic observations (Kennedy & Volz, 1985; Rao *et al.*, 1985; Ray & Payne, 1988). One of the reasons for this, may be the difficulties encountered in culturing primary

epithelial tissue and the subsequent processing of the material for studying host-parasite interactions.

A) TISSUE CULTURE

For tissue culture purposes, adult animal tissue was used because it is easier to obtain epithelial growth with adult tissue compared to embryonic tissue. The disadvantages of using adult tissue compared to embryonic tissue for primary tissue culture are that the latent period before any growth is observed often lasts for several days and the success rate for various tissues is low. In addition, the tissue is more difficult to disaggregate prior to culture. Furthermore, the tissue is usually contaminated and requires treatment to ensure sterility. One of the few advantages is that, initially, fibroblasts do not grow as rapidly as occurs with embryonic material (Paul, 1975).

Sobel *et al.* (1979) emphasized the placing of tissue immediately into cold medium and into culture within 30 min of biopsy for normal human vaginal tissue. The tissue was not subjected to enzymatic disaggregation. In this study, the tissue was placed in cold PBS with a higher concentration of antibiotics and an antimycotic than was used in the growth medium, as suggested by Paul (1975) for the treatment of contaminated tissue. The tissue was also washed with AA medium containing anti-PPLO solution to

remove any PPLO contaminants. Preliminary studies indicated that tissue did not grow unless subjected to enzymatic treatment. The tissue was treated with either trypsin or collagenase or a combination of both enzymes. The methods included trypsinization in the cold overnight, followed by trypsinization at 37C or trypsinization at 37C only. According to Paul (1975), trypsinization in the cold results in higher yields of viable cells. The method was modified by increasing the incubation period at 37C from the suggested 15-20 min to 1 h. Incubation for 15 min was insufficient and the best results were obtained for 1 h.

The hormonal status of the animal was taken into consideration when it was found that some tissue under the same conditions of treatment grew while others did not. Donor animals, each at a different hormonal status, were selected and tissue from rats in proestrus, oestrus and a single attempt of metoestrus showed high growth success rates, while a single attempt to grow tissue from the rat in dioestrus, failed. It should be noted that the proestrus phase was closer to oestrus. The reason for a greater growth success rate for tissue from animals in proestrus and oestrus may be complex, i.e., involving the effect of hormones. Alternatively there may be the simple explanation that during the latter stages of proestrus and at oestrus, there is increased activity and multiplication

of epithelial cells (Martin, 1985) which would increase the possibility of their survival in culture.

There was not much difference observed in the growth of tissue that was trypsinized in the cold compared to trypsinization at 37C, usually employed, and tissue was trypsinized in the cold out of convenience. Tissue that was trypsinized in the cold overnight and for an additional 24 h at 4C grew successfully, indicating that cold trypsinization did not destroy the tissue. A single attempt at cold trypsinization without the use of collagenase, was unsuccessful. Tissue that was trypsinized in the cold, incubated for 15 min at 37C and then treated with collagenase grew in one out of three attempts when the hormonal condition was unknown while tissue from rats in oestrus did not grow in two attempts. The reason for growth in that case was most probably due to the prolonged incubation with collagenase. Growth did not take place with trypsinization at 37C for 2 h, followed by collagenase treatment of 5 h. It was difficult to establish the reason for failure when the hormonal status was unknown. Although it cannot be stated that trypsinization on its own or prolonged trypsinization of 2 h does not result in growth, it is unlikely that it does. Trypsin is used mainly for the disaggregation of embryonic tissue and certain adult tissues such as the kidney. Because of the large amount of collagen in the tissue of adult animals, some tissue requires treatment

with collagenase, often in combination with trypsin (Paul, 1975; Wolffe & Tata, 1984).

As suggested by Owens (1976), the tissue was minced in a plastic petri dish since the scoring of the flask with the scalpel during cutting helps to keep fragments of tissue in place during the procedure. The same author treated tissue, that was inoculated into the culture flask, with collagenase only for 18 to 24 h at 37C. Thereafter, the tissue was drawn through a pipette several times to further disaggregate the material. The cell clusters were allowed to settle, while the collagenase solution was removed and replaced with medium. The cell clusters were then distributed into fresh culture flasks. Owens *et al.* (1976) used the same method to attempt primary culture of a variety of normal human tissues. Their overall success was 38% and they achieved 50% and 100% success for the growth of bladder and kidney tissue, respectively. It is interesting to note that single attempts to grow vaginal or uterine tissue were unsuccessful.

In this study, the use of collagenase only for 4 h of incubation at 37C did not result in growth. Tissue treated with collagenase at 4C overnight and incubated for 5-8 h at 37C, did not grow in both of two attempts. Overnight incubation at 37C resulted in growth of tissue obtained from rats in oestrus for both of two attempts. The tissue had disaggregated completely into clusters of

epithelial cells as well as single cells that adhered well to the culture vessel. However, growth was very poor with one or two clusters per flask giving rise to small epithelial colonies that grew slowly and to a limited size. This indicated that cells lost viability with overnight collagenase treatment.

Collagenase may be used in a mixture with trypsin and serum but some types of tissues completely lose cell viability when dissociated. A sequential dissociation method is often necessary (Paul, 1975). A combination of trypsin and collagenase treatments provided the best results. A single attempt at growing tissue that was trypsinized in the cold and then treated with collagenase overnight, was unsuccessful. Since the hormonal status of the animal was unknown, it cannot be stated with any certainty that this method will not provide results. However, it seems unlikely that cell viability would be unaffected. The sequential enzyme treatment with trypsin followed by collagenase for 2-8 h at 37C resulted in high growth success rates. Tissue from rats in proestrus which were trypsinized for 1 h at 37C and incubated with collagenase for 4-6 h at 37C showed 100% successful growth compared to the method of cold trypsinization and a 2-5 h collagenase incubation period which was only 67% successful. These results indicated that either a longer incubation period with collagenase was necessary or trypsinization at 37C was favoured compared to

trypsinization in the cold. However, a comparison of the growth success rates for tissue from rats in oestrus, that was trypsinized at 37C or in the cold and incubated with collagenase for 3-5 h and 1-4 h, respectively, showed that there was negligible difference between the two methods. Indeed, if the latter method is considered, the 1 h incubation with collagenase was unsuccessful and if not included in the comparison, showed that the success rate of growth was 7% higher than the former method. Thus, it would seem that neither cold trypsinization nor a shorter incubation period with collagenase, had any adverse effect on growth. It should be noted that the sequential enzyme treatment did not result in complete disaggregation of tissue.

Tissue that was inoculated into flasks and left to dry for 1 h without foetal bovine serum (R. Haniff, University of Natal, personal communication), showed increased adherence to the culture flask compared to that left in serum overnight. It was found that growth was unaffected by the addition of glutamine to William's medium E. Sobel *et al.* (1979) used the medium with glutamine for the culture of human vaginal epithelium. The cells were obviously capable of converting the L-glutamic acid provided to L-glutamine. This is surprising since a number of established mammalian cell lines appear to have a high requirement for glutamine. Glutamine was synthesized from L-glutamic acid when high concentrations of the latter was

present (Paul, 1975). However, William's medium E contains a low concentration (50 $mg.l^{-1}$) of L-glutamic acid.

William's medium E is a complex synthetic medium with a relatively moderate to high concentration of glucose compared to most other media. This medium was originally formulated for the growth of adult rat epithelial cells for long term cell cultures. William's medium E contains 2 $g.l^{-1}$ glucose which is low compared to Dulbecco's modified Eagle's medium (DMEM). The latter medium contained no sodium pyruvate compared to the former medium. Although the amino acid content was similar, DMEM contained higher concentrations of individual amino acids. William's medium E contains $CuSO_4$, $MnCl_2$, $ZnSO_4$, ascorbic acid, biotin, ergocalciferol, α-tocopherol, vitamins A, B_{12} and K,in addition to the inorganic salts and vitamins found in DMEM. Owens *et al.* (1974) used DMEM supplemented with 10% foetal calf serum and 10 $\mu g.ml^{-1}$ insulin to grow normal liver, ovary, skin and mammary gland epithelium. A non-essential amino acid mixture was added to the supplemented medium by Owens *et al.* (1976) who were unable to grow normal human vaginal tissue. In this study, the medium was supplemented with 20% foetal bovine serum (R. Haniff, University of Natal, personal communication) and LPSR-1 to provide additional growth factors. Preliminary experiments showed that LPSR was necessary for the growth of the tissue. It appears that adult rat vaginal tissue

has exacting requirements for growth. However, Sobel *et al.* (1979) was able to grow human vaginal tissue by supplementing William's medium E with 10% foetal bovine serum. In addition to the lower serum concentration, they did not use any additional growth-promoting factors. One of the reasons for this difference may be that rat vaginal tissue is more responsive to hormones compared to human tissue, by the very nature of the ovarian cycle. This may also have influenced the growth of tissue obtained from animals during various phases of the cycle. Alternatively, the quality of the serum may not have been acceptable for the growth of this particular tissue. However, it seems likely that vaginal epithelial tissue has complex nutritional requirements and that broad spectrum media such as DMEM, which are used for a variety of mammalian cell lines, may not be acceptable for this type of tissue. The use of 5-7% CO_2 in air was sufficient for the growth of the tissue. Sobel *et al.* (1979) used 5% while Owens (1976) used 10% CO_2.

Epithelial tissue outgrowth was initiated after a long lag period of 5 to 7 d. Monolayers of tissue resembled squamous epithelium *in vivo*. Cultures were viable at 6 weeks of culture and exfoliated cells were visible after about 3 weeks of culture, suggesting multilayering of tissue at certain points in the culture. Fibroblasts presented a problem since they tended to restrict epithelial growth. Fibroblastic growth was controlled by

short periods of trypsinization, as suggested by Owens (1976), on a weekly basis which was sufficient to remove fibroblasts but leave epithelial tissue relatively undisturbed. The epithelial cultures were allowed to establish themselves and were only trypsinized after 10 d of growth. It was suggested by Gilbert & Migeon (1975) that a medium substituting D-valine for L-valine would allow for the growth of epithelium, while suppressing fibroblastic growth. White *et al.* (1978) were able to grow primary mammary epithelium without fibroblast contamination by substituting D-valine for L-valine in DMEM. William's medium E contains L-valine and it would be interesting to determine whether fibroblastic growth would be inhibited if the L-amino acid was replaced with D-valine. Sobel *et al.* (1979) did not find it necessary to trypsinize cultures because contamination with fibroblasts was very slight. The reason was that they were able to separate the epithelium from the underlying connective tissue. Preliminary experiments were unsuccessful when the growth of epithelium, separated from underlying tissue, was attempted in this study. The advantage of using normal cultured tissue as the host model for *C. albicans* interactions is that *in situ*, it is representative of the *in vivo* host system as opposed to exfoliated donor cells or cultured cell lines. As is the situation with cell lines, it provides a controlled environment for interactions compared to the use of donor

cells which display donor to donor as well as day to day variability (Sandin *et al.*, 1987).

The epithelial cells were as difficult to dissociate as they were to grow. The proteolytic enzyme trypsin was recommended for the dissociation of cells in a number of cell lines (Paul, 1975). The enzyme protease, was used by Owens (1976) to dissociate epithelial cells which were released from the culture flask as sheets of cells. The tissue fragments were pipetted to produce smaller clusters which were used mainly for subculturing purposes. For part of this study it was necessary to dissociate the tissue into single cells without causing much damage to the cell membrane, which is the site for host-parasite interactions. Since the host receptor for *C. albicans* may be a glycoprotein (Skerl *et al.*, 1984), the use of proteolytic enzymes for cell dissociation was out of the question. However, tissue was trypsinized at 37C for 2 min for a minimum of 24 h before the interaction to remove any fibroblasts. Preliminary experiments were carried out using a non-enzymatic dissociation medium which contained sodium citrate, HEPES buffer and sodium chloride, as suggested by White *et al.* (1978). The tissue was incubated in dissociation medium for 1 h at 37C. The cells did not detach completely and a "policeman" was used to loosen tissue that was still adherent.

A commercial non-enzymatic dissociation solution, the ingredients of which were not made available, was used to dissociate cells for the adhesion assay. An incubation period of 5 min followed by the use of a "policeman" was sufficient to remove the material. For VEC, a longer incubation period was not advisable since the tissue was difficult to dissociate, even after 20 min, without the use of a "policeman". With the BEC, however, it was easier to dissociate cells which did not require the aid of a "policeman". According to Paul (1975) chelating agents, such as EDTA, may be used for non-enzymatic dissociation of cells. Since a lengthy incubation period was not used, it is unlikely that the presumptive epithelial cell receptors for *C. albicans* were destroyed. The tissue, which was dissociated into clusters of cells, was drawn through a 20 G needle a few times to further dissociate cells. Remaining undissociated cell clusters, larger than 60 µm in diameter, would have blocked the particle size analyzer sensor. For this reason, it was necessary to filter the cell suspension through 58 µm mesh size Nytex. The final cell concentration was low and cells grown from the same animal were pooled for that reason. The disadvantages of growing and dissociating primary epithelium obviously are greater than the advantage of using a controlled system of normal cells for this type of host-parasite interaction.

B) QUANTIFICATION OF ADHESION

The reasons why the cells were fixed prior to counting was that no further changes in the cells could occur. The advantage of determining two particle sizes for both yeast and epithelial cells was that it indicated the ratio of cell sizes in a cell suspension. The overall percentage adherence of YC to epithelial cells was low (Fig. 14), with the highest values obtained for cultured VEC, viz., 9% and 5%, as determined by particle size analysis and visually. Similarly the lowest value of 1% was obtained for non cultured VEC for both assay methods. The values obtained using particle size analysis were higher compared to the visual assay. King *et al.* (1980) also obtained different values for percentage YC adherent to exfoliated VEC, using different assay methods. These authors used the same ratio of 1000:1 YC to epithelial cells, as was used in this study. However, they observed higher adherence with the visual count compared to a radiometric assay. The latter value was 9% while the former was 18%. A comparison of the percentage adherence values of adherent YC to exfoliated cells, determined visually, is shown in Table XVIII. Although higher values of percentage adherence were obtained by King *et al.* (1980) compared to this study, the adherence was 7% lower for BEC compared to VEC. This concurs with the finding in this

TABLE XVIII. A comparison of the percentage adherence of exfoliated epithelial cells, as determined by a visual count

EPITHELIAL CELL	YC : EPITHELIAL CELL	ADHERENCE (%)	AUTHORS
BEC	20:1	30	Collins-Lech *et al.*, 1984
Corneocytes	20:1	20	Collins-Lech *et al.*, 1984
BEC	50:1	18	Ghannoum & Elteen, 1987
BEC	500:1	36	Kimura & Pearsall, 1978
BEC	1000:1	11	King *et al.*, 1980
VEC	1000:1	18	King *et al.*, 1980
BEC	5:1	44	Sandin *et al.*, 1982
VEC	100:1	14	Segal *et al.*, 1984
VEC	25:1	86	Sobel *et al.*, 1982

study that the adherence of YC to VEC was greater by 3% and 2% compared to BEC, as determined by particle size analysis and the microscopic count, respectively (Fig. 14). However, Sandin *et al.* (1987) found that YC adherence to BEC from 10 subjects was higher compared to VEC (Fig. 1), with one exception. The differences between the results of this study and that carried out by King *et al.* (1980) may be due to the difference in assay

parameters, viz., the interacted suspension of cells was shaken at 200 rpm for 30 min of incubation by King *et al.* (1980). Since the material was incubated for 90 min, in this study, the percentage adherence should be higher because of the increased incubation time (Cox, 1983; Gorman *et al.*, 1986; Kimura & Pearsall, 1978; Kimura & Pearsall, 1980). Thus, it would seem that shaking the interacted suspension was of importance. Alternatively, the host cells may differ in their affinity for *C. albicans*.

Although it was found that adherence increased with an increasing YC to epithelial cell ratio in individual studies (Cox, 1983; Kimura & Pearsall, 1978; King *et al.*, 1980), the results of different studies shown in Table XVIII are contradictory since they indicate, with a few exceptions, that the lower the YC to epithelial cell ratio, the higher the percentage adherence. The differences observed in the adherence values in Table XVIII may be attributed to the differences in assay parameters. The highest adherence was obtained for VEC by Sobel *et al.* (1982). These authors used the tissue culture medium, William's medium E, as the assay medium as well as a prolonged incubation of 2 h. While Kimura & Pearsall (1978) found that adherence increased with the use of a tissue culture medium, TC199, King *et al.* (1980) did not find any significant difference between the use of PBS or TC199 as the assay medium. A relatively high

percentage adherence of YC to BEC was obtained by Sandin *et al.* (1982). In the latter study, the lowest YC to epithelial cell ratio, viz., 5:1, was used (Table XVIII). The interacted cells were filtered through a 12 µm membrane filter and washed with 250 times the initial volume, the highest compared to any other study. Centeno *et al.* (1983) found that washing with 10 times the initial volume was sufficient to remove non-adherent YC. A wash volume of 2,5 times the initial suspension was used by Ghannoum & Elteen (1987) and could be the reason why the percentage adherence was low, considering the initial YC to epithelial cell ratio. In this study, the interacted cell suspension was washed with 20 times its volume and considering that the initial YC to epithelial cell ratio was high, may have been insufficient, resulting in low percentage adherence values. However, Gorman *et al.* (1986) interacted YC with exfoliated BEC at a ratio of 100:1, with shaking, at 37C for 90 min and using a Coulter counter to determine particle size, obtained the low percentage adherence of 9%. Microscopic counts confirmed these results. One of the reasons why the visual count was lower than the particle size count in this study, may be the difficulty in counting large numbers of adherent YC because of the high initial ratio of YC to epithelial cells and the subjectivity involved. A further disadvantage is that it is a lengthy and tedious procedure to count the number of cells sufficient for statistical purposes. A high visual count may be obtained if YC that

coadhere are included in the count. The advantages of the visual count is that the YC coadherence and the adherence of YC to individual epithelial cells can be observed. The advantage of a count based on particle size is that actual counting time is rapid. The disadvantage, however, is that the material has to be treated so as to avoid particulate contamination as well as sensor blockage. The cell count in this study was obtained by an indirect method, i.e., the number of non-adherent YC was determined relative to the initial YC concentration and the percentage adherent YC thereby calculated. An indirect determination is subject to error which was minimised by the use of a control.

Non-cultured VEC from the rat had the lowest percentage of adherent YC for both the particle size and visual counts (Fig. 14), as well as the least adhesive capacity (Table XVII). The latter property was described by Segal *et al.* (1984) as the ability of epithelial cells to bind 20 or more YC. Non-cultured VEC were obtained by the overnight treatment of tissue with collagenase, which most probably resulted in the loss of cell viability and damage to the host cell surface. The latter would be the most likely reason since YC are capable of adhering to desquamating non-viable cells. The adhesive capacity of cultured VEC as well as BEC was high (Table XVII), indicating that the integrity of the cell membrane was retained and the non-enzymatic dissociation procedure was relatively mild

compared to enzymatic methods. However, the overall low percentage adherence obtained indicated that cell dissociation may alter or damage the host cell surface to a certain extent. If a tissue culture host model is to be used, it appears that *in situ* observations of the host-parasite interaction would be advisable compared to dissociation of host cells.

1. Adherence Inhibition Studies

The adherence inhibition studies indicated that 100% loss in the adhesive capacity of both VEC and BEC occurred with mannose, conA and *E. coli*, with the exception of *E. coli* treatment for BEC which resulted in a 94% reduction (Table XVII). The percentage adherence of YC to epithelial cells was significantly reduced by the same treatments, as determined by both particle size and visual counts (Tables XV and XVI). The highest reduction in adherence was obtained with conA treatment for BEC in both assays. Similar results were obtained with mannose and conA treatment for VEC. Treatment with mannose resulted in a lower reduction for BEC, while treatment with *E. coli* showed the least reduction for both BEC and VEC. However, inhibition of adherence by *E. coli* for VEC, determined visually, was similar to that observed with mannose and conA treatments. The results of conA and mannose treatments concur with the findings of Sandin *et al.*

(1982). These authors found that YC adherence to BEC decreased to a similar extent with conA and to a lesser extent with mannose treatments. However, the treatment of BEC or corneocytes with conA or mannose did not have any significant effect of YC adherence (Ray *et al.*, 1984). After 1 h of incubation a dose-dependant decrease followed by an increase in adherence at 3 h was observed (Fig. 7). The authors suggested a bimodal response to mannan over time. Lee & King (1983), using similar concentrations of mannan, found that there was no difference observed in adherence. However, they interacted cells for 30 min, which may have been too short a time to observe any difference.

Centeno *et al.* (1983) found that mannose decreased YC adherence to BEC and to a lesser extent, uroepithelial cells. It is interesting to note that the latter authors and Sandin *et al.* (1982) interacted cells for 1 h which may account for the decreased adherence observed. In this study, an incubation time of 90 min was used, as well as a higher concentration of mannose, which may account for the increased reduction in adherence.

Collins-Lech *et al.* (1984) found that mannose had no significant effect on adherence of YC to BEC and corneocytes. The reason for this observation may be that extremely low concentrations of the sugar were used. Makrides & MacFarlane (1983) observed that conA and

mannose increased adherence of YC to HeLa cells. The latter effect, although slight, was also noted by Reinhart *et al.* (1985) for YC adherence to VEC. The latter authors used a radiometric assay method with an incubation period of 90 min. There was no decrease in adherence with any of the 10 sugars and amino-sugars tested at high concentrations. The authors were unable to explain their findings, and attributed the difference in results compared to other studies to the difference in assay technique including the method used to separate adherent from non-adherent YC. Makrides & MacFarlane (1983) suggested that mannose may not be directly involved in candidal adherence, but that mannose receptors on the surfaces of HeLa cells and YC may be linked together by conA, explaining the increased adherence observed with conA treatment. The authors attributed the difference in their results for conA compared to the decreased adherence observed by Sandin *et al.* (1982) to the fact that the latter authors pretreated BEC with conA rather than having added the lectin to the cells prior to incubation. However, decreased adherence was observed in this study although conA was added to the cell suspension prior to incubation. Makrides & MacFarlane (1983) obtained results contrary to other studies, probably because of the different host model, viz., HeLa cells, used. Sandin (1987) found that conA was capable of reducing adherence by 63% if added before 20 min of incubation, after which adherence was not significantly affected. The author

suggested that a two phase adhesion process of YC to BEC occurs which is irreversible after a given time. Thus it appears, from this study as well as a number of the studies reviewed using mannose and conA adherence blocking experiments, that mannose is essential or at least required for part of *C. albicans* adherence to epithelial cells.

The mechanism of adhesion for *C. albicans* was investigated in this study by the use of blocking agents in the adhesion assay. The sugar under study, mannose, may represent either adhesin or receptor analogue, since mannose-containing moieties on both yeast and host cell surfaces may be involved in adhesion. The use of sugars in adherence inhibition tests is based on their ability to competitively inhibit adherence. The use of adhesins or adhesin analogues is based on the same principle. The use of sugars in blocking experiments resulted in the same observation as this study (Sandin *et al.*, 1982), while Centeno *et al.* (1983) indicated that mannose may comprise the host cell receptor. However, L-fucose (Sobel *et al.*, 1981) or amino sugars and not mannose, were suggested to comprise the epithelial cell receptor (Collins-Lech *et al.*, 1984; Segal *et al*, 1982) or the YC adhesin (Segal *et al.*, 1982). According to Critchley & Douglas (1987b), the amino-sugar N-Ac-D-glucosamine may be responsible for binding to *C. albicans*. They suggested that the latter two sugars and, perhaps, mannose may function as the

receptor depending on the strain of *C. albicans* involved. A pathogenic strain of *C. albicans* was also found to interact via fucose-containing receptors (Douglas, 1987). On the basis of partial inhibition of adherence by L-fucose of *C. albicans* GDH2346, it was suggested that the natural epithelial receptor is larger than L-fucose and/or that a specific stereochemical configuration is necessary. The possibility exists that additional adhesion mechanisms are required (Critchley & Douglas, 1987b)

Mannan was unable to significantly inhibit adherence (Lee & King, 1983; Ray *et al.*, 1984) as well as mannose or mannoprotein (Lee & King, 1983). However, the use of proteases and reducing agents, decreased adherence, indicating that the protein portion of the adhesin was essential to adhesion. A mannoprotein adhesin was isolated by McCourtie & Douglas (1985b) and purified by Critchley & Douglas (1987a). Chemical and enzymatic treatments to destroy the carbohydrate moiety, did not significantly affect adherence compared to destruction of the protein portion of the adhesin, which resulted in partial or total reduction of adherence.

Lectins, especially conA, have also been used as blocking agents in adherence assays. This is achieved by steric interference or competitive inhibition of adhesion. In this study, conA was responsible for the highest reduction in adherence. This was also noted by Sandin *et al.* (1982)

while Sandin (1987) observed a time-dependant effect. However, Makrides & MacFarlane (1983) and Critchley & Douglas (1987b) found that adherence was increased and attributed this to the ability of conA to bind to *C. albicans* as well as epithelial cells, thereby enhancing adhesion. The reason proposed for the different results was that Sandin *et al.* (1982) and Sandin (1987) used germinated YC fixed with formaldehyde. It was suggested that conA would be able to bind but not link cells fixed with aldehyde (Rotrosen *et al.*, 1985). The glycoprotein, Fn, has also been implicated as the receptor for *C. albicans* (Skerl *et al.*, 1984) as well as certain bacteria (Espersen & Clemmensen, 1982).

2. Role of Bacteria

The effect of bacteria on candidal adherence is unclear. In this study, adherence was significantly decreased upon *E. coli* treatment by as much as 92 and 81% for VEC, determined by visual and particle size counts, respectively. A lower reduction was observed for BEC, especially for the particle size count. Cox (1983) found a 2% increase in YC adherence to BEC from patients receiving antibiotic therapy for 2 d. The increase in adherence was statistically significant because of the small standard deviation. It was suggested that bacteria compete with *C. albicans* for glucose in the *in vivo* environment, thereby controlling growth and adherence of YC to host tissue. Since this is unlikely to occur *in*

vitro, a decrease in adherence, as observed in this study, may be caused by the physical interference of bacteria. Kennedy & Volz (1985) suggested that intestinal bacteria may physically inhibit adherence of *C. albicans*. Alternatively, bacteria may compete with *C. albicans* for binding sites or modify the sites on epithelium (Larsen *et al.*, 1977; Sandin *et al.*, 1987). Sobel *et al.* (1981) found that precoating VEC with vaginal isolates of lactobacilli decreased candidal adherence. In this study, *E. coli* showed greater preference for VEC rather than BEC, indicating that the host cell type, probably by virtue of the receptors for *E. coli* that were expressed, determined the extent of inhibition. This would further suggest that this organism competed for the same binding sites as *C. albicans* which were expressed to a lesser degree on BEC (which probably was the reason why the adherence of YC to VEC was greater than to BEC). Relatively recently, the use of lactobacilli, instilled intravaginally, was successful in preventing recurrent urinary tract infections (Bruce & Reid, 1988). The latter study indicated the importance of competition among the microbial flora in preventing infection. However, it was found that the increased presence of bacteria in the rat vagina *in vivo* did not prevent candidal colonization (Larsen & Galask, 1984). Similarly, Andermont *et al.* (1983) found that enteric bacilli, including *E. coli*, did not inhibit growth of *C. albicans* in the gut of conventional mice.

In vitro studies by Centeno *et al.* (1983) and Makrides & MacFarlane (1983) indicated that piliated bacteria enhanced candidal adherence. It was suggested that these bacteria mediate YC adherence by a bridging mechanism, i.e., by linking receptors on the surfaces of *C. albicans* and epithelial cells. While no such observation has been made *in vivo*, it may be possible that certain bacteria enhance adherence while others are inhibitory. It would be interesting to note whether high counts of bacteria as well as which types of organisms occur in patients with candidiasis. Carlson & Johnson (1985) observed non-introduced bacterial rods together with *C. albicans* colonizing mouse oesophagus, 10 d after fungal inoculation. The relationship between the organisms was not determined. The same authors observed a mutualistic relationship between *S. aureus* and *C. albicans*, where the fungus promoted systemic murine infection by the bacteria, probably by modifying the environment to promote infection. The adhesive capacity of epithelial cells was reduced by as much as 100% for VEC which also suggests that the bacteria occupied or modified *C. albicans* binding sites. Thus it would seem that a complex situation occurs *in vivo* and *in vitro* experiments on their own are insufficient to provide explanations of microbial interactions. Studies on the effect of microbes on vaginal candidiasis are complicated by the fact that the indigenous microbial flora is under hormonal control.

Ryley (1986) indicated that the pH of the human vagina is acidic compared to the neutral pH of the rat, and as such contains different microorganisms, a fact which should be taken into consideration when comparing the effect of vaginal bacteria on *C. albicans* in humans and in the rat.

The discrepancies observed with the results may be attributed to the various differences in assay parameters for the determination of adherence mechanisms. An important factor would be the type of host cell employed. Most of the information pertained to exfoliated BEC (Centeno *et al.*, 1983; Collins-Lech *et al.*, 1984; Cox, 1983; Gorman *et al.*, 1986; Kimura & Pearsall, 1978; King *et al.*, 1980; Ray *et al.*, 1984; Sandin, 1987; Sandin *et al.*, 1982) and to a lesser extent VEC (King *et al.*, 1980; Lee & King, 1983; Reinhart *et al.*, 1985; Segal *et al.*, 1982; Sobel *et al.*, 1982). The reason may be that it is relatively convenient to obtain BEC from donors compared to VEC. Sandin *et al.* (1987) found that exfoliated host cells differed in their ability to bind *C. albicans*, which indicated that it may be a reason for the differences in results observed. Not only did donor differences exist, but day to day variation as well. One would expect the latter for VEC considering the hormonal implications (Segal *et al.*, 1984) but it was also true for BEC. The need for a controlled host model led to the use of cultured tissue, in its undissociated form. A HeLa cell line was used by Makrides & MacFarlane (1983). The

problem with using a cell line is that the results obtained may not be a true reflection of the interaction with normal cells. Decreased adherence was observed with the buccal cell line in this study compared to VEC. The reasons may be that these BEC were transformed cells, BEC are inherently less adhesive than VEC, or a longer incubation with dissociation medium compared to VEC resulted in a loss of adhesive ability. Rotrosen *et al.* (1985) and Sobel *et al.* (1982) used primary normal human cultured tissue. The former author studied the *C. albicans* interaction with endothelium while the latter used multilayer vaginal tissue. The latter authors experienced problems in the adherence assay because of the multilayered nature of the tissue. Since this study concentrated on the use of dissociated cells, only a relative comparison can be made with previous studies. Cell dissociation may have resulted in damage to cell receptors, accounting for the overall low percentage adherence. This was probably true for non-cultured cells from the rat, which were subjected to prolonged enzymatic treatment and subsequently showed a reduced adherence capacity compared to the cultured counterpart.

C) YEAST CELL SURFACE

Apart from the use of blocking agents or enzymatic and chemical treatments to investigate mechanisms of adherence, yeast growth conditions were manipulated to either increase (McCourtie & Douglas, 1984; 1985a;

1985b) or decrease the synthesis of adhesin (Douglas & McCourtie, 1983). In the latter instance, it was found that the sugar-containing moiety of the adhesin was important in adherence, since the inhibition of mannoprotein glycosylation resulted in decreased adherence. The growth of YC in the stationary phase resulted in the production of a mannoprotein adhesin, the expression of which depended on the carbon source (McCourtie & Douglas, 1984). Galactose-grown YC produced optimal amounts of adhesin, followed by YC grown in sucrose. Both galactose and sucrose were present at high concentrations in the medium. The growth was relative to that of YC grown in medium containing a low concentration of glucose. The chemical composition of the adhesin produced in response to the different carbon sources, was similar and immunologically identical. However, strain differences in the adhesin were observed, since adhesin produced by *C. albicans* GDH2023 was capable of inhibiting adherence for the same strain but not for *C. albicans* GDH2346 (McCourtie & Douglas, 1985b). The adhesin was fibrillar in nature and produced on the YC surface (Tronchin *et al.*, 1984).

1. TEM

A fibrillar layer, stained with ruthenium red, was observed on the yeast cell surface (Fig 22). Ruthenium red was used to stain polysaccharide-containing material on the YC surface which is otherwise difficult to

demonstrate (Marrie & Costerton, 1981). This fibrillar layer probably mediates adhesion to epithelial cells since it was observed in close association with cultured VEC (Fig 21). Marrie & Costerton (1981) found that a ruthenium red positive matrix was involved in the adherence of *C. albicans* to BEC from patients with oral candidiasis. Barnes *et al.* (1983) observed a fibrillar outer cell wall layer mediating adhesion to endothelial cells of rabbits with experimental renal candidiasis. Similarly, Tronchin *et al.* (1984) and Persi *et al.* (1985) found that surface fibrils appeared to mediate adhesion to exfoliated BEC and VEC, respectively. The fibrils of *C. albicans* may be analogous to bacterial pili since fimbriae, measuring 1-3 µm, were reported to be present on two isolates of *C. albicans* including strain GDH2346. The presence of fimbriae was not peculiar to *C. albicans* but was common to many species of basidiomycetous and ascomycetous yeasts. It was suggested that these yeast species, have a surface that is adapted for common cell surface functions. The fimbriae, however, may also be multifunctional (Gardiner *et al.*, 1982). It would be interesting to observe whether other animal pathogenic fungi possess the same feature. An inference from the above finding is that *C. albicans* has not evolved any specific mechanism to promote adherence. Garcia-Tamayo *et al.* (1982) also observed the ruthenium red stained outer cell wall layer of YC and pseudohyphae outside and within VEC from patients with vaginal candidiasis. Although SEM

demonstrated the presence of germ tubes associated closely with the host cell (Fig. 17) penetration of the latter was not shown. However, TEM observations indicated a YC partly surrounded by dense material of host cell origin (Fig. 19). Upon closer investigation of this material, the presence of disorganized membranous components were revealed (Fig. 23). It appears that the yeast penetrated a superficial host cell of the tissue which was multilayered at that point. The cell was probably in the process of desquamation and the host tissue was further traumatized by the YC.

2. SEM

The YC showed preference for exfoliated host cells as observed by SEM (Fig. 15). Rao *et al.* (1985) found that YC adhered in large numbers to damaged epithelial cells, as well as the stroma of the cornea, compared to the occasional adherence of YC to intact superficial corneal epithelium. *C. albicans* showed preference of adherence to the subendothelial extracellular matrix exposed by contracted cells compared to confluent monolayers (Klotz & Maca, 1988). The YC tended to coadhere (Fig. 20) which was also observed by King *et al.* (1980). It was suggested by these authors that coadherence may allow *C. albicans* to accumulate sufficient YC at certain areas of host tissue, *in vivo*, in order to overcome host defence mechanisms.

The SEM of the interaction showed that YC formed a cavity in the epithelial cell at the site of contact for cells *in situ* (Figs. 16a and 16b), but not for dissociated VEC (Fig. 18). Ray & Payne (1988) observed cavitation of newborn murine corneocytes *in situ*, by *C. albicans*. The multilayered nature of cells *in situ* would allow for the observation of this phenomenon, as compared to dissociated cells. As in this study (Fig. 16b), Ray & Payne (1988) observed strand-like amorphous material, which was referred to as "cohesin". This material linked YC to the host cell and was not found to be an artefact because of its absence in a number of control situations in the experiment. It was suggested that this material possibly contained polysaccharides, was of host origin and participated in the adherence of *C. albicans*. Extracellular strand-like material was also observed by Persi *et al.* (1985) with SEM of the *C. albicans* interaction using VEC on membrane filters. In addition, these authors also observed what they referred to as "burrowing" of the YC in the host cells. Ray & Payne (1988) inhibited the production of keratinolytic acid proteinase in *C. albicans* and found that the cavity formation was inhibited, although adherence and cohesin formation was unaffected. The authors suggested that the proteinase contributes to invasion of host tissue. In this study TEM indicated a YC residing in a depression at the site of contact (Fig. 19). This was not observed in TEM by Marrie & Costerton (1981) for YC that were tightly

adherent to BEC. Thus it appears that tissue culture represents conditions similar to that *in vivo* and it is possible that the cavity formation observed was due to the production of fungal enzymes. Rotrosen *et al.* (1985) showed that *C. albicans* was phagocytosed into the cultured endothelial cell indicating that initial entry of the YC in the host cell is not an invasive process. This finding may be attributed to the property of endothelium and would hardly be representative of oral and vaginal infection. However, Klotz *et al.* (1983) using a high YC concentration found that extensive destruction of cultured endothelial cells occurred with penetration by *C. albicans*.

Although, in this study, a well-developed RER system was observed in the cultured tissue (Fig. 24), no membranous network was observed in the host cell at the YC-host cell interface. It does not appear that any recognition is involved in this host-parasite relationship. It does not seem likely that the adhesion of *C. albicans* to epithelial cells is highly specific, since it is indiscriminate in its attachment to mammalian host tissue. As is true of opportunistic pathogens, the organism takes advantage of changes in the host environment to colonize host tissue and if not opposed by an adequate host defence system, would establish infection. Although the role of the microbiol flora is unclear, in the healthy individual it must undoubtedly participate in preventing candidal

colonization. Damaged or ageing cells may express the receptor for *C. albicans* which is thought to contain either a fucose, amino sugar or D-mannose moiety. A multifunctional glycoprotein, such as Fn, may be involved in binding *C. albicans*. The yeast adhesin is probably a mannoprotein and both sugar and protein components appear to be necessary for adhesion. The adherence of *C. albicans* appears to be a means whereby the organism can physically attach and stabilize itself. By doing so it then commits itself to a morphological change in form which enhances invasion of host tissue and provides a mechanism to elude the host defence system. In addition, proteolytic enzymes are produced as an invasive measure. Ray *et al.* (1987) observed a correlation between acid proteinase production and phenotype switching of certain *C. albicans* strains. *C. albicans* was capable of high frequency reversible switching to seven colony phenotypes (Slutsky *et al.*, 1985). Soll (1988) found that *C. albicans* was capable of switching at the site of infection in patients with vaginal candidiasis and it was suggested that switching may provide the organism with the means to invade the various body tissues and/or with increased avidity. Alternatively it may control the organism's response to the changing environmental conditions such as those present in the vagina. *C. albicans* is a successful pathogen because of its complexity, which is as yet, not understood. Obviously the adherence of the organism to host tissue is of importance in controlling the disease,

since inhibition of adherence would prevent subsequent colonization and infection.

To conclude, the main objective of this study was to develop a normal tissue culture host model system. Monolayer as well as multilayer outgrowths of normal vaginal epithelium were obtained which provided a controllable *in vitro* host system. The host model was particularly useful for electron microscopic observations of the *C. albicans*-host cell interaction, which showed that the ruthenium red-stained fibrillar outer layer of the YC wall appeared to mediate adherence of YC to each other and to the host cell membrane. *In situ* observations were similar to those observed in previous *in vivo* studies compared to the use of exfoliated host cells. Dissociation of epithelial cells for quantifying adherence was successful. It may, however, have led to damage to the host cell receptors resulting in an overall low percentage adherence. While particle size analysis provides a rapid method for quantification, the indirect procedure may be subject to error. The microscopic counting method, by virtue of its popularity, appears to be the method of choice, since the advantages of observing the interaction visually, outweigh the disadvantages. Adherence inhibition studies indicated significant reduction with mannose and conA for both methods of quantification. These results indicated that the YC adhesin and/or the host cell receptor for this isolate of

C. albicans contained a mannose component. The addition of *E. coli* resulted in decreased adherence, indicating that this organism competes with *C. albicans*. The results of previous studies are contradictory, with some in agreement with those obtained in this study. The discrepancies may be attributed to the differences in methods and assay parameters. Mannoprotein appears to be accepted as the YC adhesin, which is thought to be a fibrillar component residing on the outermost cell wall layer. The nature of the host cell receptor is unclear, although sugars or amino-sugars may comprise, in part, the receptor depending on the strain of the pathogen. In view of the differences observed in the studies on the host-parasite interactions of *C. albicans* using variable donor host cells, there is a need for the controlled host system of normal tissue culture; this would be suitable for investigative ultrastructural studies but has limitations concerning the quantification of adherence.

SUMMARY

Normal primary rat vaginal epithelium was successfully cultured by subjecting tissue to sequential enzymatic disaggregation. The tissue was either trypsinized in the cold overnight followed by incubation of 1 h at 37C or incubated for 1 h at 37C only. This was followed by fragmentation of the tissue which was then treated with collagenase for 2 to 8 h at 37C. After inoculation into tissue culture flasks the material was incubated for 1 h at 37C, followed by incubation in William's medium E in an atmosphere of 5-7% CO_2 in air. Epithelial outgrowths appeared after about 5 d of incubation. Fibroblastic overgrowth was prevented by weekly trypsinization for 2 min at 37C commencing after 10 d of culture. Multilayered tissue was visible after 3 weeks of culture.

Cultured VEC and BEC cell lines were dissociated using a commercial dissociation medium. Non-cultured VEC from the rat were obtained by overnight treatment with collagenase at 37C. The host cells were interacted with *C. albicans* YC for 1 h at 37C. The percentage adherent YC, according to particle size counts was 9%, 6% and 1% compared to that obtained with the visual method, i.e., 5%, 3% and 1% for cultured VEC, BEC and non-cultured VEC, respectively. Adherence inhibition studies with mannose (100 $mg.ml^{-1}$) and conA (150 $\mu g.ml^{-1}$) resulted in decreased adherence for both VEC and BEC. A 95% and 93% reduction was observed

with mannose for VEC on the basis of particle size or visual counts, respectively. Similarly, VEC displayed a 92% decrease in adherence with conA for both assay methods. The adherence of BEC was reduced by 95% and 97% with conA and by 90% and 83% with mannose as determined by particle size and microscopic counts, respectively. The adhesive capacity of VEC and BEC, measured as the ability of epithelial cells to bind 20 or more YC, was inhibited by 100% by both mannose and conA treatments. These results indicate that mannose is essential for the adhesion of this *C. albicans* isolate to epithelial cells. *E. coli* was found to decrease VEC adherence to *C. albicans* by 81% and 92% and BEC adherence by 56% and 80% as determined by particle size and visual counts, respectively.

SEM of the interaction *in situ*, showed increased adherence of YC to exfoliated cells compared to the tissue monolayer. Extracellular material on the host cell surface was observed in association with *C. albicans*. Cavity formation of the epithelial cell was observed at the site of YC contact. The implications of these findings in the pathogenesis of the organism are discussed. *In situ* TEM showed the ruthenium red outer cell wall layer of *C. albicans* in close association with the host cell membrane. This fibrillar layer was also seen in association with a coadhering YC. The fibrils, which are most probably mannoprotein in nature, may

mediate adherence of *C. albicans* to epithelium and each other.

This study provided a suitable controlled *in vitro* host model to investigate the host-parasite interaction of *C. albicans*. The dissociation of cultured epithelium is not recommended for this purpose and *in situ* observations may indicate conditions *in vivo*.

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