This is to certify that the

dissertation entitled

ANALYSIS OF ADHESIVE, CELL SURFACE, AND COLONIZING PROPERTIES OF CANDIDA ALBICANS

presented by

MICHAEL JAMES KENNEDY

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Botany and Plant Pathology

Major professor

Date 11/10/88

ANALYSIS OF ADHESIVE, CELL SURFACE, AND COLONIZING PROPERTIES OF *CANDIDA ALBICANS*

By

MICHAEL JAMES KENNEDY

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1988

ABSTRACT

ANALYSIS OF ADHESIVE, CELL SURFACE, AND COLONIZING PROPERTIES OF *CANDIDA ALBICANS*

By

MICHAEL JAMES KENNEDY

The ability of *Candida albicans* to attach to host mucosal surfaces and colonize the gastrointestinal tract may play an important role in pathogenesis. The present studies examined the adhesive properties of *C. albicans* and the interaction between this fungus and the intestinal microflora. Adhesion of *C. albicans* to buccal epithelial cells (BECs) and plastic was significantly modified by altering growth and assay parameters, particularly growth medium. Significant differences in adhesion to BECs and plastic were noted when *C. albicans* was grown in the same complex medium from different manufacturers and in different lots of medium from the same manufacturer. Electron microscopy also revealed significant differences in surface topography and cell wall ultrastructure of *C. albicans*. At least three different adhesins were observed to exist on the surface of *C. albicans* that mediate its

adhesion to BECs: (i) a cell wall adhesin, (ii) an adhesin located in an outer floccular layer, and (iii) an adhesin expressed below the surface that becomes evident when the outer cell wall layer is thinned. The ability of white and opaque phenotypes of C. albicans to attach to BECs and plastic were also examined. "White cells" were found to be significantly more adhesive to BECs than "opaque cells". No difference in adhesion to plastic between the two phenotypes was noted, although opaque cells were twice as hydrophobic. The adhesion of C. albicans to plastic was further analyzed by binding isotherms, Langmuir isotherms, and Scatchard plots. Collectively, the results suggest that there are at least two distinct mechanisms of adhesion of C. albicans to BECs and plastic. The interaction of C. albicans with intestinal bacteria was examined in continuous-flow culture models. The suppression of C. albicans by intestinal bacteria was collectively regulated by the depletion of nutrients and anaerobiosis, in addition to the production of inhibitory substances by intestinal anaerobes (e.g., short-chain fatty acids). The intestinal microflora apparently controlled C. albicans collectively by several mechanisms, but Candida cells could become tolerant to some of these mechanisms and survive at a low levels in the gut probably by associating with GI mucosal surfaces.

DEDICATION

To my loving wife and daughters
Laura Ann Kennedy
and
Alyssa Lauren Kennedy and Andrea Michelle Kennedy
whose love and companionship
have blessed my soul.

An additional expression of love
is extended to my
parents and grandparents
for their constant love,
support, guidance and prayer.

"For God so loved the world, that
He gave His only begotten Son, that
whosoever believeth in Him should
not perish, but have everlasting life."
(John 3:16)

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor, Dr. Alvin L. Rogers, for his friendship, support, and guidance during the years I have studied and trained in his laboratory. He has been an excellent mentor and has provoked me to expand my knowledge of medical mycology and mycology. It has been a privilege to have trained in his laboratory, learned mycology under his direction, and have him as a friend. I also respect the professionalism by which he has conducted his courses and laboratory.

I would also like to thank Drs. Everett S. Beneke, Karen L. Klomparens, Ronald J. Patterson, and Paul A. Volz for serving on my guidance committee. I thank all of the above professors for their example of sound scientific concepts and principles, and for many helpful comments and stimulating discussions. I especially thank Dr. Paul A. Volz for stimulating my early interest in medical mycology, and for his friendship over the past ten years.

My special thanks to Dr. Robert J. Yancey, Jr. for his encouragement and endorsement of the research described herein, and for his helpful ideas, comments, criticisms, and stimulating discussions. I also thank him for his friendship and encouragement.

I thank my wife, Laura Ann Kennedy, for her love and support, her help in proofreading the manuscript and checking that all the references were in order. I also thank my wife and children for their patience as I was away from home, and especially for allowing me to postpone house duties during the final few months of my studies and research. Most of all I thank my wife for staying up with me when I worked through the night, and for sharing just the right verses of scripture (Isaiah 40:28-31) when I was very discouraged.

Finally, I would like to thank all of my family for their constant love, encouragement, support, and prayers throughout all of my years of undergraduate and graduate study. I thank my mother for endeavoring to teach me professional qualities and for a godly heritage in Christ.

This work was supported in full by The Upjohn Company.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Candida albicans is commonly found residing in the oral cavity, gastrointestinal (GI) tract, and female genital tract of humans (Rippon, 1988). The yeast is usually commensal, but in predisposed persons clinical infection can occur as a result of its opportunistic nature. In patients who are compromised immunologically and undergoing prolonged antimicrobic therapy, *C. albicans* can reach high numbers in the GI tract and, subsequently, pass through the intestinal mucosa to initiate systemic infection by the hematogenous route (Myerowitz *et al.*, 1977), particularly in patients with acute leukemia (Meunier-Carpenter *et al.*, 1981; Myerowitz *et al.*, 1977; Ray, 1980). Similarly, colonization of the GI tract by *C. albicans* may also lead to involvement in a number of other disease syndromes (Odds, 1988; Rippon, 1988).

Although the determinants of colonization and dissemination of *C. albicans* from the oral cavity, esophagus, and GI tract have not been completely defined, it is likely that adhesion and association of *Candida* cells with host mucosal surfaces and prosthetic devices plays an important role (Kennedy, 1988; Rotrosen *et al.*, 1986). For instance, it is unlikely that colonization of various sites of the alimentary tract, such as the small intestine, could take place in the face of the rapid

passage of material and fluid through the tract due to peristalsis, unless these organisms could associate with the mucosa. Moreover, prevention of Candida mucosal association by bacterial antagonism has been shown to reduce oral and GI colonization and dissemination by this fungus (Kennedy and Volz, 1985a; Liljemark & Gibbons, 1973). Considering further the sloughing of epithelial cells and the bathing action of fluids over host mucosal surfaces and various tissues, and the fact that Candida lesions may arise in virtually any tissue of the body, suggests that adhesion is also an important determinant of thrush and denture stomatitis, vaginitis, endocarditis, mucocutaneous candidosis, and renal Candida infections.

Because of the importance of Candida adhesion and colonization to human health, then, there can be no reasonable doubt that it would be of great benefit to clinical medicine if adhesion and colonization by C. albicans could be regulated in such a way that the beneficial effects are retained, and the detrimental effects are eliminated. This will only be possible, however, when the adhesive properties of this organism are characterized at the molecular level and when the ecologic mechanisms that control C. albicans in the gut are more thoroughly understood. To that end, the research described herein was undertaken to analyze adhesive properties of C. albicans and to examine interactions between this fungus and the intestinal microflora.

REVIEW OF THE LITERATURE

Candida and candidal infections

The genus Candida is comprised of a heterogeneous collection of asporogenous yeasts that reproduce by budding and belong to the form-class Deuteromycetes and the form-family Cryptococcaceae (Rippon, 1988). Some members of this genus have sexual stages belonging to the genus Leucosporidium in the Ascomycetes (Kreger-van Rij, 1984). There is also some evidence that C. albicans has characteristics that are common to both Basidiomycetes and Ascomycetes, but classification in either of these "perfect" classes will have to await the isolation of the teleomorph state (if there is one) of the organism.

Candida albicans is similar to other members of the genus in that nearly all species have a predominant morphologic form that is unicellular, with budding as the predominant form of reproduction. However, only C. albicans produces germtubes when grown in serum at 37°C for one or two hours (Cooper & Silva-Hutner, 1985) and is the predominant species to produce chlamydospores when grown on chlamydospore agar or corn meal agar to which Tween-80 has been added (Beneke & Rogers, 1980; Kreger-van Rij, 1984). Assimilation and fermentation patterns for carbohydrates, biotype testing, and molecular and serological techniques have also been applied to the

taxonomy of Candida species (Kreger-van Rij, 1984; Odds, 1985). Although some of these tests are still far from routine clinical use for yeast identification, they have provided a great deal of information regarding the antigenic similarities and relatedness of various Candida species.

Jonsen (1955), for instance, first showed antigenic cross-reactivity among Candida species due to similarities in surface polysaccharides a little over thirty years ago. Studies by others have since confirmed this and have classified Candida species into six groups according to their complements of heat stable and heat sensitive antigenic components (Murray & Buckley, 1966; Segal et al., 1975; Sweet & Kaufman, 1970; Tsuchiya et al., 1961). The existence of several common antigenic components has likewise been observed with fluorescent antibody and agglutination reactions to both soluble and insoluble antigenic extracts (Dastidar, 1968; Geck & Novak, 1967; Gordon et al., 1967; Hasenclever, 1961; Kemp & Solotorvsky, 1964). Furthermore, all of the so-called pathogenic Candida species possess at least one common antigen, and most posses two (Tsuchiya et al., 1961). It is apparent, then, that there are considerable similarities between antigens from the major opportunistic Candida pathogens. In fact, several investigators have shown that C. albicans, C. stellatoidea and C. tropicalis possess as many as seven common antigens, whereas C.

guilliermondi, C. krusei, C. parapsilosis and C. pseudotropicalis appear to be antigenically distinct (Tsuchiya et al., 1961).

The existence of two antigenic types among strains of C. albicans, moreover, has been demonstrated by several investigators (Hasenclever, 1965; Hasenclever & Mitchell, 1961 a&b; Hasenclever et al., 1961; Summers et al., 1964). One antigenic type, serotype A, possess the same number of antigens as the other type, serotype B, plus at least two additional antigens (Hasenclever & Mitchell, 1961 a&b; Hasenclever et al., 1961). Antigenic similarities between type A C. albicans and C. tropicalis, and type B C. albicans and C. stellatoidea have also been observed (Hasenclever & Mitchell, 1961a&b; Hasenclever et al., 1961; Murray & Buckley, 1966; Stewert-Tull et al., 1966; Sweet & Kaufman, 1970). Two-dimensional immunoelectrophoresis of soluble cytoplasmic extracts from C. albicans and C. tropicalis, for instance, indicate that these two strains may share as much as 70% of common antigens (Odds, 1988). A third antigenic type of C. albicans, namely serotype C, has also been proposed by Muller and Kirchoff (1969), however, there have been no other studies to date to either confirm or reject such a group for C. albicans (Odds, 1988).

One common antigenic factor for Candida species, and other yeast (e.g., Saccharomyces cerevisiae) appears to be cell wall polysaccharide, which is often found in cross-

reacting antigenic extracts (Hasenclever, 1965; Kemp & Solotorvsky, 1964; Stewart-Tull, 1966). As noted by Odds (1988), there is good evidence to suggest that antigenic specificity in mannans and glucans (the primary make-up of yeast cell walls; see below) from Candida species is predominately due to the nature of glycosidic linkages in side branches from the main polysaccharide chains (Suzuki & Sunayama, 1969; Yu et al., 1967 a&b), and that mannan structure is primarily responsible for the distinction between type A C. albicans/C. tropicalis and type B C. albicans/C. stellatoidea (Kolarova, et al., 1973; Odds, 1988). Proton magnetic resonance (PMR), furthermore, showed similar spectra for mannans isolated from serotype A & B C. albicans, C. tropicalis, and C. stellatoidea, while PMR spectra were quite distinct for mannans from C. guilliermondi, C. krusei, C. parapsilosis and C. pseudotropicalis (Spencer & Gorin, 1969).

Electrophoresis of soluble extracts from broken Candida cells showed that there were at least 46 identifiable proteins from the extracts, and that there were characteristic similarities and differences between the protein patterns for several Candida species (Shecter et al., 1972). Such experiments, have confirmed the close similarities between C. albicans and C. stellatoidea, and that strains of C. tropicalis appeared to exhibit extreme dissimilarities between this species and other Candida species (Odds, 1988; Shecter et al., 1972). Likewise,

others have also reported that C. albicans and C. tropicalis showed quantitative differences with respect to phosphatase enzymes (Odds & Gonzales, 1974; Saugar et al.,1975) and several oxidases (Odds, 1988), but that both species uniquely produced an intracellular transglucosyl amylase (Sawai & Hehre, 1963). Measurements of the quanine plus cytosine (G + C) content of Candida DNA showed a very wide range of base compositions among members of the Candida genus (from 35 to 50% G + C), but that the DNA base compositions of C. albicans, C. tropicalis and C. stellatoidea were very similar (Stenderup & Bak, 1968). In contrast, such similarities were not seen in DNA-DNA hybridization studies between C. albicans and C. tropicalis which showed a relative homology of only 8%, whereas C. albicans and C. stellatoidea exhibited a homology of over 80% (Bak & Stenderup, 1969). Finally, C. guilliermondii, C. parapsilosis and C. pseudotropicalis have been shown to be capable of photoreactivation after ultraviolet irradiation, while strains of C. albicans, C. krusei, C. stellatoidea and C. tropicalis were not (Miller & Sarachek, 1974).

It appears, therefore, that although a wide variety of taxonomic studies have shown that there are a number of similarities between C. albicans, C. stellatoidea and C. tropicalis, there is no tendency for the species of Candida that are pathogenic to form a taxonomic cluster

that would serve to distinguish them from the so-called nonpathogenic Candida. One must assume, then, that Candida species frequently found to cause disease in debilitated patients, and C. albicans in particular, possess one or more virulence mechanisms that render them pathogenic to their mammalian hosts. For instance, C. albicans can produce a number of nonspecific hydrolytic enzymes, which are probably responsible for damage to host tissues in cases of candidosis. Kapica and Blank (1957) reported that C. albicans could produce keratinase, an enzyme that allows fungi to attack keratinized tissues. The production of several peptidases, proteases and phospholipases have also been demonstrated and studied in detail (Odds, 1988). MacDonald and Odds (1983), moreover, recently showed a direct relationship between virulence for mice and protease production in C. albicans. A correlation relationship between phospholipase and proteinase activity and the adhesion of C albicans and other Candida species has also recently been made (Barrett-Bee et al., 1985; Ghannoum & Elteen, 1986).

Candida infections have been reported in virtually every tissue of the human body (Odds, 1988; Rippon,1988). Different forms of candidosis, therefore, have been referred to in the past generally as either "superficial" or "systemic" candidosis. This is primarily due to the fact that the oral cavity and GI tract have been considered to be continuations of the body surface.

However, since there are important differences in cutaneous and mucocutaneous involvement, both histologically and pathologically, infections of the alimentary tract will not be considered as superficial in this discussion. The various clinical forms of candidosis, then, may be listed according to the primary tissue system involved: cutaneous, mucocutaneous, and systemic (Rippon, 1988).

The normal adult usually has a high resistance to infections by C. albicans (Rogers & Balish, 1980). Candida infections are, therefore, uncommon unless there are alterations in host defenses or predisposing environmental conditions, even though C. albicans can live as a commensal on several body surfaces (e.g. the oral cavity, GI tract and vagina). Factors which can predispose an individual to candidosis appear to be many and varied, an can be placed into four general groups: natural (e.g., digressions from normal hormonal or physiologic status), dietary, mechanical (e.g., bowel resection), and iatrogenic medical factors (e.g., drug treatments) (Odds, 1988). From this list, as well as numerous publications on experimental animal infections and clinical observations, it appears that there are two major deterrents to infection by C. albicans that are disrupted by such factors: (i) suppression of Candida colonization by the indigenous host microflora and (ii) an intact immune system (Odds, 1988).

"Thrush". Oropharyngeal and esophageal thrush has been known since at least the time of Hippocrates, even though C. albicans was not discovered until about 1841 (Odds, 1988; Rippon, 1988). The disease, which is the most common form of candidosis, is most prevalent among infants, but may be seen in patients of all ages. Recently, chronic oropharyngeal and esophageal candidosis has been noted as a first sign of clinical AIDs (Rippon, 1988). Thrush is characterized by the presence of white patches that appear as discrete lesions on the surfaces of the buccal mucosa, throat, tongue, and gum linings. The lesions develop to become confluent pseudomembranes that resemble milk curds, which can be scraped off with difficulty to reveal a raw, erythematous base (Odds, 1988). The infection is not usually painful, although in severe cases erosion and ulceration of the mucosa may be seen (Jenkins, 1973; Ludlam & Henderson, 1942; Marples, 1968; Winner & Hurley, 1964). While oral thrush is the best-known and most common form of oral candidosis, C. albicans also has an important role in the etiology of denture stomatitis, angular cheilitis, leukoplakia, and possibly other types of oral disease (Epstein et al., 1984; Odds, 1988; Rippon, 1988). It should be noted that oral thrush has seldom been attributed to yeast species other than C. albicans (Epstein et al., 1984; Odds, 1988).

Howlett and Squier (1980) in an experimental model of Candida-host interactions, and Wilborn and Montes (1980;

Montes & Wilborn, 1980) in patient sstudies, recently characterized the colonization and invasion of oral epithelium using scanning and transmission electron microscopy. From these studies, it was found that attachment of Candida cells to the superficial cells of the oral mucosa appeared to involve an intimate contact between the epithelial surface and layers of the fungal cell wall. Further examination showed the involvement of a number of layers of the cell wall of C. albicans within the epithelium, and that changes were evident in the organization and definition of the outer cell layers in budding yeast-phase cells and in hyphae and pseudohyphae participating in colonization and invasion of the epithelial cells. In other words, yeast-phase cells were closely associated with the epithelium, while hyphae and pseudohyphae invaded and penetrated into the epithelium. During hyphal penetration, a close seal was maintained between the invading hyphae and the surrounding epithelial cell envelope. No other damage to the epithelial cell surface was noted except at the site of entry. Within the epithelium there was only occasional loss of cytoplasmic components, these being in the vicinity of the invading hyphae. This would suggest that there was an enzymatic lysis associated with the invasive process, and that this enzymatic lysis was localized, possibly at the hyphal tip. Candida infections of oral mucosa, then, may be considered as occurring in three phases: (i) mucosal association and

(Liljemark & Gibbons, 1973); (ii) hyphal invasion of the epithelial cells and establishment of an intracellular habitat (Montes & Wilborn, 1968), which may be initiated by a tropism to the host tissue by C. albicans (Howlett & Squier, 1980; Wilborn & Montes, 1980); and (iii) the provocation of an inflammatory response in underlying tissues (Howlett & Squier, 1980; Montes & Wilborn, 1968; Wilborn & Montes, 1980).

The major source of infections for infants has often thought to be the mother's vagina during birth, where C. albicans can reside as a commensal (Rippon, 1988). Other sources have also been implicated, including infected feeding bottle nipples (Odds, 1988; Winner & Hurley, 1964), the skin of nurses and mothers (Jennison, 1966; Ludlam & Henderson, 1942; Odds, 1988; Winner & Hurley, 1964) and the air or dust of nurseries (Jennison, 1966; Odds, 1988). Among adults, oropharyngeal and esophageal thrush may appear as a complication of infection or medical care resulting from a number of clinical situations, since C. albicans is known to reside unnoticed in the oral cavity in approximately 20 to 50% of healthy individuals (Odds, 1988; Winner & Hurley, 1964).

Gastrointestinal candidosis. Candida infections of the stomach, small intestine and colon have been reported more frequently in recent years, but have been described far less often than cases of oropharyngeal and esophageal thrush or related diseases. Winner and Hurley (1964) have

reviewed the case histories prior to 1962, but there have been only a few descriptive reports since. C. albicans and other Candida species (as well as Torulopsis glabrata) have been isolated from samples of gastric ulcers, and have been suggested to interfere with the healing of the ulcers (Gotlieb-Jensen & Anderson, 1983; Thomas & Reddy, 1983). Likewise, C. albicans has been shown to invade the intestinal mucosa of the small intestine and colon (Joshi et al., 1981; Rippon, 1988). Endoscopic examination and biopsy specimens, for instance, have demonstrated the presence of Candida organisms in ulcerative lesions and plaques (Joshi et al., 1983; Kozin & Taschdjian, 1962). Macroscopically chronic gastrointestinal candidosis appears as two types: (i) the mucosa may be covered by a thick, shaggy, cream-colored confluent membrane (similar to that seen in oral thrush), which strips away easily to reveal grayish-brown plaques of confluent ulceration; or (ii) there may be more localized, discrete and scattered lesions, which may be few or many and may occur in any part of the gut (Rippon, 1988; Thomas & Reddy, 1983). The latter type of lesion or plaques may be grayish-brown or cream-colored raised plaques or areas of shallow ulceration, and may be covered by a rough friable brown exudate; thus making the second type of lesion very hard to observe endoscopically (Joshi et al., 1981). Biopsy specimens have shown that invasion of the mucosa can be accompanied by the downward, parallel growth of mycelial

elements (Koznin & Taschdjian, 1962; Rippon, 1988).

In view of the frequent presence of Candida organisms in the gut, it has often been suggested that GI Candida infections are endogenous in nature (Odds, 1988). However, since C. albicans frequently inhabits the gut of healthy, asymptomatic individuals as commensals (in low numbers), it follows that host resistance mechanisms must first be disrupted before C. albicans can initiate invasion and infection of host tissues. One of the most important factors that can predispose an individual to oral and/or GI candidosis and, subsequently, systemic candidosis, is a partial or complete disruption of the indigenous microflora (Odds, 1988). The oral and intestinal microflora protects the host against mucosal association, colonization and invasion of oral and GI mucosal surfaces (Liljemark & Gibbons, 1973; Kennedy & Volz, 1983; Kennedy & Volz, 1985 a&b).

Systemic candidosis. Systemic Candida infections are an important medical complication in seriously debilitated patients (Meunier-Carpenter et al., 1981; Myerowitz et al., 1977; Ray, 1980), and are important causes of morbidity and mortality among patients who are compromised immunologically or undergoing prolonged antibiotic therapy (Odds, 1988; Ray, 1980; Rippon, 1988). Although Candida cells may be introduced into the bloodstream by a number of exogenous sources (including contaminated i.v. catheters and infected wounds), numerous authors consider

the direct passage of viable C. albicans through the GI mucosa into the host bloodstream to be the most important mechanism leading to systemic candidosis (Krause et al., 1969; Myerowitz et al., 1977; Stone, 1974; Stone et al., 1973,1974). Factors which result in an overgrowth of C. albicans in the gut (such as prolonged antibiotic therapy), then, may render the host more susceptible to systemic Candida infections. Such infections may arise as localized infections, with only one organ affected, or as disseminated infections as the result of Candida cells being spread by the bloodstream to a number of organs (Odds, 1988; Rippon, 1988). The most important factor determining the extent and severity of such infections are the underlying host immune system, the virulence of the organism and the amount of organisms entering the host bloodstream (Odds, 1988; White et al., 1979). Cancer patients who are being treated with immunosuppressive drugs, broad-spectrum antibiotics, and/or intensive radiation therapy are the most susceptible individuals to systemic candidosis (Bodey & Rodriquez, 1975; Meunier-Carpenter, 1981; Myerowitz et al., 1977; Young et al., 1974).

Candida albicans cell wall composition, ultrastructure, and possible adhesins

The cell wall of C. albicans is a multilayered structure approximately 100 - 300 nm thick whose

appearance varies with cell age, morphologic form and culture conditions (Cassone et al., 1979; Kulkarni et al., 1980; McCourtie & Douglas, 1981; Poulain et al., 1985). Because of the putative importance of cell wall ultrastructure and surface composition in Candida adhesion and pathogenesis, the chemical composition of the cell wall of C. albicans has been studied in great detail (Cabib et al., 1982; Chattaway et al., 1968; Gooday, 1977; Kessler & Nickerson, 1959; Poulain et al., 1985; Reiss, 1986). Biochemical studies of the cell wall of C. albicans indicate that it is composed mainly of glucan and mannan polysaccharides, with lesser amounts of chitin, protein and lipid (Chattaway et al., 1968; Kessler & Nickerson, 1959; Yu et al., 1967 a&b). The presence of glucose and mannose in C. albicans polysaccharides has been a consistent finding (Chattaway et al., 1968; Jonsen, 1955; Kemp & Solotorvsky, 1964; Odds, 1988; Stewart-Tull et al., 1966), and glucan and mannan have often been found combined with protein in cell wall extracts as glucoproteins, mannoproteins and glucomannoproteins (Kemp & Solotorvsky, 1964; Kessler & Nickerson, 1959). The finding that mannan and glucan represent about 75 - 85% of the dry matter of Candida cell walls, and that the remainder of the cell wall is composed of lesser amounts of chitin, protein, and lipid suggests that it is very similar to the cell wall of Saccharomyces cerevisiae (Ballou, 1976; Chattaway et al., 1968; Kessler &

Nickerson, 1959; Poulain et al., 1985; Reiss, 1986).

Cell wall composition. Chemical analyses of the cell wall composition of C. albicans have shown that mannan makes up nearly 35 - 40% of the total dry weight of the cell wall (Bartnicki-Garcia, 1973). C. albicans mannans contain mainly α-1-6- and α-1-2-linked mannopyranose residues and much less phosphorus than those reported for S. cerevisiae (Bishop et al., 1960; Skil et al., 1964; Stewart & Ballou, 1968; Suzuki & Usuyama, 1971; Yu et al., 1967 a&b), while rare α-1-3-linkages between mannose residues have also been reported (Odds, 1988; Stewart & Ballou, 1968; Suzuki & Usuyama, 1971). The chemical structure of this polysaccharide, then, is an α-1-6-linked mannan backbone to which sidechains of up to six hexose units long are joined by α-1-2- and rare α-1-3-linkages. The phosphate molecules associated with C. albicans cell wall mannans appear to be linked together by phosphodiester bridges (Elorza et al., 1985). Analysis of mannans isolated from serotypes A and B of C. albicans has revealed differences between the mannans of these serotypes. The differences between mannans of C. albicans serotype A and B, as well as C. stellatoidea and C. tropicalis, appear to be due to the extent of branching, the length of side chains (serotype A is thought to have longer side chains than serotype B [Okubo et al., 1979; San-Blas, 1982]), and the proportion and position of α-1-3-bonds in the side chains (Odds, 1988; Stewart &

Ballou, 1968; Suzuki & Fukazawa, 1982). The impact these differences may have on Candida adhesion is presently unknown.

Glucan appears to be the most abundant polymer in the cell wall of C. albicans (Bishop et al.,1960; Elorza et al., 1983; Gopal et al., 1984; Shephard et al., 1984; Sullivan et al., 1983), and is considered to be the essential component of the "microfibrillar skeleton" (Gopal et al., 1984; Poulain et al., 1985). Glucan is a highly branched polysaccharide that consists of a backbone of β-1,6-linked glucose residues to which side chains of β-1,3-linked glucose residues are attached (Bishop et al., 1960; Yu et al., 1967). Recent studies suggest that there may actually be two types of glucan found in the cell wall of C. albicans: either β-1,3-linkages or β-1,6-linkages, both of which are highly branched (Gopal et al., 1984).

Glucoprotein, mannoprotein, and glucomannoprotein complexes have also been isolated from the cell wall of C. albicans (Kessler & Nickerson, 1959; Kolaraova et al., 1973). These molecules are all more or less tightly associated with one another and with glucan and mannan (Kolarova et al., 1973), and may serve as necessary adhesive links between glucan and mannan (San-Blas, 1982). The proteins that are complexed with mannan and glucan, in yeast phase cells, are thought to be present as water-soluble proteins and water-insoluble glycopeptides (Kolarova et al., 1973). Mannan-protein complexes are

thought to be linked through an N-acetylglucosamine residue and side chain amino groups (Odds, 1988). Glucan is also thought to be complexed to chitin (polymer of N-acetylglucosamine joined by β-1,4-linkages) adjacent to the plasma membrane (Gopal et al., 1984). Although chitin helps to anchor glucan to the cell membrane, it comprises only a small part (<1%) of the cell wall composition by dry weight (Chiew et al., 1980; Poulain et al., 1985; Sullivan et al., 1983). Most (90%) of the chitin has been shown to be associated with bud scars of yeast phase cells (Bacon et al., 1966), and the remainder forms the link between the cell membrane and the innermost insoluble glucan possibly by a mixed β-1,3-/β-1,6- linkage (Gopal et al., 1984). All of these molecules are antigenic (Isenberg et al., 1963; Ponton & Jones, 1986b; Poulain et al., 1985; San-Blas, 1982; Smail & Jones, 1984; Sundstrom & Kenny, 1984), and some (e.g., mannoproteins) may serve as Candida adhesins (McCourtie & Douglas, 1985a; Sandin et al., 1982).

The protein content of the cell wall of C. albicans has been studied less extensively than the polysaccharide content. Protein may, in some instances, make up to about 30% of the cell wall content (Chaffin & Stocco, 1983; Chattaway et al., 1968; Kobayashi & Suginaka, 1984). It had generally been thought that nearly all of the proteins found in the outer layers of the cell wall of C. albicans were associated with large mannan molecules (Poulain et

al., 1985). However, recent studies from several investigators do not support this view (Ponton & Jones, 1986a). Ponton and Jones (1986a) found that many proteins isolated from the cell wall of C. albicans had negligible mannose content. These authors further suggested that there may actually be a complex latticework of proteins situated in the outer layers of the cell wall of both yeast- and mycelial-phase organisms, and that inter- and intra-disulfide bonds in these proteins are important in maintaining the structure of this latticework.

The ability of C. albicans to exclusively produce hyphae among Candida species has led to a number of studies on cell wall ultrastructure of yeast-mycelial conversion and its relationship to infection. A number of investigators, for instance, have shown that C. albicans hyphae are: (i) usually seen in smears, scrapings or histological sections from lesions due to infection by C. albicans; (ii) often found in feces or from infected patients urine; and (iii) that C. albicans yeast-phase cells produce hyphae in host tissues within a few hours of inoculation to experimental animals (Odds, 1988). Thus, it has frequently been assumed, implied or suggested that hyphal formation by C. albicans enhances its ability to penetrate and infect host tissues (Balish & Phillips, 1966 a&b; Blyth, 1959; Cantrell & Widra, 1964; Phillips & Balish, 1966). In fact, it has often been suggested that the hyphal form of C. albicans alone is "pathogenic" or

"invasive" (reviewed in [Odds, 1988]). However, while the hypothesis that hyphal formation is the primary factor that confers an enhanced pathogenic ability on C. albicans (or that such an ability is the only factor known to correlate with the fact that C. albicans is the most common yeast pathogen) has considerable logical appeal, the fact remains that yeast-phase cells and hyphae are usually seen together in vivo. Furthermore, there have been relatively few instances in which the hyphal form of C. albicans alone has been observed in infected tissues, whereas yeast-phase cells of C. albicans only are often observed in specimens from infected patients and animals. On the other hand, it is likely that the ability to form hyphae in infected tissues is probably influenced (and may be stimulated) by local environmental factors, and may allow C. albicans to avoid removal from these tissues. For instance, the ability to form germtubes affords C. albicans an enhanced ability to attach to host epithelial cells, form emboli, and to resist ingestion by host phagocytes (Kennedy, 1988; Odds, 1988; Richardson & Smith, 1981), and the ability to form hyphae has been shown to prolong survival within leukocytes and macrophages (Baine et al., 1974; Odds, 1988; Richardson & Smith, 1981).

Nevertheless, although it has been shown that formation of hyphal forms is important in the pathogenesis of infections by C. albicans (Sobel, 1984), the question of which form is more pathogenic may never be settled.

Studies related to the mechanism of morphogenesis have shown that numerous environmental factors can influence C. albicans growth form, but the exact mechanism(s) responsible for C. albicans morphogenesis has yet to be discovered (Odds, 1988). Several studies, however, have shown dramatic changes in the cell wall composition and ultrastructure of C. albicans during the transformation from yeast to hypha (Chattaway et al., 1968; Odds, 1988; Yamaguchi et al., 1971). Such studies do suggest that the cell wall of C. albicans is synthesized in two phases: (i) first an immature, soft, primary wall is laid down, and (ii) then it is subsequently rigidified (Odds, 1988).

Analyses of the cell wall from C. albicans yeast-phase cells and mycelium clearly indicate that there are differences in the cell wall composition of these two forms and that these differences are primarily quantitative and not qualitative (Bianchi, 1977; Braun & Calderone, 1978; Cassone et al., 1984; Chattaway et al., 1968& 1976; Elorza et al., 1985). Chattaway et al. (1968), for instance, found that chitin was present at levels three times higher and protein at levels nearly three times lower in the mycelial form. Bianchi (1967) found twice as much lipid in hyphae as in blastoconidia, and three times as much carbohydrate has been found in hyphal membranes as in yeast membranes (Shwartz & Larsh, 1980). Recent studies confirm this and found that mycelial forms were more active than yeast forms in the

accumulation of steryl esters and triacylglycerols (Ghannoum et al., 1986b). Furthermore, it was also shown that yeast lipids contained much larger proportions of free sterols than the mycelial lipids, whereas the mycelial lipids contained several times more sterols than the yeast forms (Ghannoum et al., 1986b). Other differences in lipid composition from yeast and mycelial forms were also noted in that study.

Chemical analyses indicate further that there are quantitative differences in hexoses, hexosamines, and amino acids of purified cell wall preparations from blastospores and pseudohyphae (Chattaway et al., 1968 & 1976), which may be related to changes in the activities of glycolytic enzymes at the branch points for polysaccharide synthesis from hexoses in the glycolytic metabolic pathway (Chattaway et al., 1968 & 1976). Quantitative differences of several hydrolytic and glycolytic enzymes between the two forms have also been reported (Odds, 1988; Shawartz & Larsh, 1980).

Cell Wall Ultrastructure. The ultrastructure of the cell wall of C. albicans has been examined by several investigators using a variety of procedures to reveal the precise location of the principle wall components (Cassone, 1973; Cassone et al., 1978; Djaczenko & Cassone, 1971; Poulain et al., 1981). Several cell wall layers have been identified for C. albicans (Poulain et al., 1985). These layers have been labeled LO to L8 Poulain et

al. (1985). Cytochemical staining (Djaczenko & Cassone, 1971; Poulain et al., 1981) revealed that each layer contains a dominant polysaccharide (Poulain et al., 1985). The gross appearance of the cell wall as viewed by electron microscopy is that of a sandwich structure with an outermost set of layers of high electron density (L0 to L4), an inner layer of high electron density (L8), and an intermediate set of layers of lower electron density (L5 to L7) (Poulain et al., 1978, 1981, & 1985). It should be stressed that the gross appearance of the cell wall has been shown to be directly influenced by the growth parameters used for preparation of the test cells (Cassone et al., 1973; Cawson & Rajasingham, 1972; Djaczenko & Cassone, 1971; Garrison, 1981; Montplaisir et al., 1976; Poulain et al., 1978, 1981, & 1985). Poulain and co-workers (Poulain et al., 1978, 1981, & 1985) found that both the growth medium and the age of the cells affected the number and appearance of layers in the cell wall. Recent studies have shown further that other growth parameters (e.g. temperature) can also have a significant effect on cell wall ultrastructure (McCourtie & Douglas, 1981). Moreover, some of these changes, particularly in the outermost layers, have been shown to correlate with the adhesiveness of C. albicans to both biological and nonbiological surfaces (Douglas et al., 1981; McCourtie & Douglas, 1981 & 1984). This will be described in more detail below.

Adhesins. Although there have been limited ultrastructural observations on the mechanisms of C. albicans adhesion, such studies do suggest that at least two morphologic classes of Candida adhesins exist: floccular and fibrillar (Kennedy, 1988). The floccular adhesins are present as a thick (approximately 100 to 400 nm) "fuzzy" cell wall coat and appear to be somewhat amorphous (Tronchin et al., 1984). Occasionally, this material appeared to have an ordered alignment around the cell wall (Tronchin et al., 1984), whereas at other times it was unevenly distributed on the cell surface (McCourtie & Douglas, 1981) or localized only at an adhesive junction (Howlett & Squier, 1980; Marrie & Costerton, 1981). Ultrastructural studies of the adhesion of C. albicans to oral (Calderone et al., 1984; Howlett & Squier, 1980; Marrie & Costerton, 1981; Montes & Wilborn, 1968; Tronchin et al., 1984), urinary (Marrie & Costerton, 1981), and vaginal epithelium (Calderone et al., 1984) showed the floccular structures to be present on yeast in infected tissues, and that they may indeed mediate Candida adhesion. Using tissue explants, for instance, Howlett and Squier (1980) found that a floccular outer layer mediated the adhesion of C. albicans to oral mucosal cells. Candida yeast phase cells harvested from media with high concentrations of certain carbohydrates have been found to be highly adhesive, and contained an outermost floccular layer that was absent from

blastospores that were significantly less adhesive which were cultivated in media without these carbohydrates (McCourtie & Douglas, 1981).

The fibrillar material (called elsewhere fimbriae [Gardiner et al., 1982]), in contrast, can be seen on the cell surface as thin filamentous structures arranged perpendicularly to the cell surface and evenly distributed around the entire cell (Barnes et al., 1983; Lee & king, 1983a). Lee and King (1983a) viewed the fibrillar structures on the cell surface of C. albicans mediating adhesion to renal endothelium. The diameters of these structures have not been measured, but appear to be within the size range (2 to 10 nm) of fimbrial adhesins of bacteria (Jones & Isaacson, 1983). Likewise, the length of the fibrillar adhesins has not been accurately measured, but these appear to be less than 0.5 μm long (Lee & King, 1983a), considerably shorter than bacterial fimbriae (Jones & Isaacson, 1983). Fibrillae also have been observed to mediate the adhesion of C. albicans to other surfaces such as epithelial cells (Marrie & Costerton, 1981; Rotrosen et al., 1985; Tronchin et al., 1984). Both types of adhesins stain with ruthenium red (Marrie & Costerton, 1981), which has an affinity for anionic polymers such as polysaccharides (Luft, 1961).

The precise chemical and organizational nature of these two morphologic structures is not known. It is very likely, however, that these structures represent distinct

adhesive entities. There is some evidence to suggest that C. albicans does produce more than one adhesin (Douglas, 1985a; Kennedy et al., 1987; Sandin, 1987a). It should be noted that the presence of one or both types of structures on the cell surface of C. albicans does not, by itself, indicate that the structures play a role in adhesion (Kennedy, 1988). Candida cells devoid of floccular or fibrillar structures have been shown to be highly adhesive to buccal epithelial cells (BECs) (Kennedy, 1988). Furthermore, not all known microbial adhesins can be identifiable as discrete surface structures (Jones & Isaacson, 1983).

Various studies have also suggested that two cell wall components primarily act as adhesins for C. albicans (Douglas, 1985 a&b; Rotrosen et al., 1986). It has been suggested, for instance, that a mannose containing moiety, probably a mannoprotein (McCourtie & Douglas, 1985a), on the surface of C. albicans is the Candida adhesin (Sandin & Rogers, 1982; Sandin et al., 1982). However, analogous studies also have suggested a possible role for cell wall chitin as the adhesin (Segal et al., 1982). Lipids also have been suggested to play a role in adhesion to epithelial cells (Ghannoum et al., 1986a). Moreover, other studies suggest that there may be more than one adhesin (Douglas, 1985a; Kennedy et al., 1987; Sandin, 1987a), and at least one report suggests that Candida adhesion may not be mediated by a specific adhesin-

receptor interaction but may instead be entirely nonspecific attachment (Reinhart et al., 1985). Possible cell wall components that serve as Candida adhesins are listed in Table 1.

The majority of reports using lectin and carbohydrate blocking experiments have indicated that a mannoprotein on the surface of C. albicans probably serves as the adhesin in a number of adhesive reactions (Douglas, 1985 a&b, Rotrosen et al., 1986). Rossano and Tufano (1975) and Sandin et al (1982), for instance, reported that pretreatment of C. albicans with Concanavalin A, a lectin which binds to α-D-mannosyl residues, inhibited adhesion of the yeast to human BECs. Adhesion could also be inhibited by adding mannose or α-D-methylmannopyranoside (a mannose derivative) to the adhesion assay medium during the incubation period (Sandin & Rogers, 1982; Sandin et al., 1982). Addition of tunicamycin, an antibiotic which, in yeasts, specifically inhibits mannoprotein synthesis but not chitin or glucan synthesis (Kuo & Lampen, 1974), to cultures of C. albicans inhibited the formation of the floccular surface layer of C. albicans (Douglas et al., 1983). Concurrently, there was a significant decrease in adhesion of C. albicans to BECs (Douglas et al., 1981 & 1983). Moreover, Lee and King (1983b) found that extracted mannan-rich cell wall fragments of C. albicans attached to vaginal epithelial cells and that this attachment was abolished following treatment of the

Table 1. Possible cell surface components suggested to serve as *Candida* adhesins*

Possible adhesin moieties	Examples of inhibitors	References
Chitin	Glucosamine	Segal *et al.*, 1982
	Chitin-soluble extract	"
	N-acetylglucosamine	"
	Mannosamine	"
Mannan/ mannoprotein	Conconavalin A	Sandin *et al.*, 1982
	Mannose	"
	α-mannosidase	"
	D-methylmannopyranoside	"
	α-methylmannosidase	Lee & King, 1983
	Tunicamycin	Douglas & McCourtie, 1983
Glucose	Glucose	
	Glucan	
Protein	Chymotrypsin	Lee & King, 1983; Sobel *et al.*, 1982
	Trypsin	"
	Papain	Lee & King, 1982
	Pepsin	"
	Pronase	"
Lipid	Sterols	Ghannoum *et al.*, 1986

*Modified from Kennedy (1988).

fragments with α-mannosidase. This α-mannosidase selectively degraded mannan because it was free of proteases and did not contain glycosidic activity against p-nitrophenyl glucose or galactose substrate (Lee & King, 1983b). McCourtie and Douglas (19885a) showed that a crude mannoprotein preparation obtained from culture supernatants of C. albicans also associated with BECs and inhibited subsequent yeast adhesion. This extracellular polymer of C. albicans was found to contain a high mannose content (65 to 82%), and lesser amounts of protein (7%), phosphorus (0.5%) and glucosamine (1.5%) (McCourtie & Douglas, 1985a).

The studies of Maisch and Calderone (1981) suggest a role of α-mannan in the adhesion of C. albicans to fibrin-platelet clots formed in vitro (Maisch & Calderone, 1980). Sheep erythrocytes coated with an alkali-soluble cell wall extract attached to a fibrin-platelet matrix, whereas nonconjugated sheep erythrocytes did not attach. The adhesion promoting effect was abolished by pretreating the alkali extract (72% polysaccharide and less than 1% protein) with α-mannosidase before conjugation to erythrocytes (Maisch & Calderone, 1981). Similarly, alkali extraction of Candida cells significantly inhibited C. albicans adhesion to human epithelial cells (Sandin, 1987a). Likewise, α-mannan or a mannan-associated cell wall constituent may also serve as an adhesin in attachment to vascular endothelium. Rotrosen et al.

(1985) showed that Candida immune serum significantly blocked adhesion to endothelial cells, but that this activity was abolished by immunoprecipitation of immune serum with C. albicans mannan but not by similar adsorption with particulate chitin.

There is also evidence to suggest that protein alone may serve as an adhesin, or that the protein portion of the mannoprotein complex is more important than the carbohydrate moiety in mediating attachment. Adhesion of C. albicans to epithelial cells has been shown to be decreased after exposure to heat or various proteolytic enzymes (Lee & King, 1983b; Sobel et al., 1981). Pretreatment of C. albicans with trypsin, chymotrypsin or pronase reduced adhesion to fibrin-platelet clots (Maisch & Calderone, 1981). Likewise, Critchley and Douglas (1985) found that pretreatment of an extracellular polymer (thought to serve as an adhesin) from C. albicans with heat, dithiothreitol or certain proteases, but not sodium metaperiodate or α-mannosidase, either partially or completely destroyed its ability to block Candida adhesion to BECs. Similarly, a protein component from C. albicans pseudohyphae also reduced the binding affinity of pseudohyphae for neutrophils (Diamond & Krzesicki, 1978). Studies on bacterial adhesion are consistent with this view as they have shown that most of the well characterized bacterial adhesins (with only two exceptions) are proteins (Jones, 1977; Jones & Isaacson,

1983).

Other studies directed at identification of Candida adhesins using selective inhibitors of adhesion have suggested that cell wall chitin may be involved in C. albicans adhesion (Segal et al., 1982). Chitin, a hydrolysate derivative of chitin, and N-acetylglucosamine all significantly inhibited attachment of C. albicans to vaginal epithelial cells (Segal et al., 1982). Moreover, it was also observed that the latter two substances also reduced the infection rate in a rat model of vaginitis (Lehrer et al., 1983). Other investigators have not been able to reproduce these results (Sandin et al., 1982). Although it seems unlikely that such a small portion of the cell wall, which is localized only to inner layers of the cell wall or bud scars, could serve as an adhesin, it should again be noted that those investigators (Sandin et al., 1982) did not use the same growth media as that used by Segal and co-workers (1982). Further blocking experiments with chitin or "chitin-soluble extract" should be performed after growth of several strains of C. albicans on the yeast extract medium used by Segal et al. (1982), compared with that after growth on other media (Kennedy, 1988), to determine more conclusively whether or not chitin plays a role in adhesion. Another approach to determine whether chitin or other cell wall components serve as adhesins would be to isolate and characterize non-adhesive mutants of C. albicans. It should be noted

also that the above experiments do not rule out the possibility that chitin or its derivatives block adhesion by means of steric hindrance or that chitin can bind to the same receptor sites that mannoprotein adhesins also bind.

In another report, Sobel et al. (1981) showed that D- and L-fucose inhibited the attachment of C. albicans to vaginal epithelial cells. However, in these experiments the fucose was allowed to react with the Candida cells first, suggesting not that a fucose-like component serves as an adhesin but that the receptor on the epithelial cell for C. albicans may also be a glycoprotein. Reinhart et al. (1985) have also suggested that Candida adhesion to epithelial cells may be entirely nonspecific, and that such adhesion is mediated solely by hydrophobic properties (Hazen et al., 1986). It seems unlikely, however, that a nonspecific phenomenon such as hydrophobicity could account for all of the distinct adhesive activities observed for C. albicans (Kennedy et al., 1987). Studies from our laboratory suggest that specific adhesion predominates over nonspecific adhesion (Kennedy et al., 1987). Therefore, the more likely explanation for C. albicans adhesion to various biological surfaces such as intestinal epithelial cells and other microorganisms is that adhesion is mediated by two or more distinct adhesive entities, in which nonspecific interactions (e.g. hydrophobicity) may or may not play a role. There is

already evidence to suggest that more than one adhesin may be involved in attachment to epithelial cells by C. albicans. However, the alternative proposition that many of the adhesive activities observed for C. albicans result from a single multifactoral adhesive cell wall component cannot be ruled out from studies to date.

Adhesion and association mechanisms

The association of C. albicans with both biological and nonbiological surfaces has been suggested to play an important role in host colonization and pathogenesis. Data has been presented on the adhesion and association of C. albicans with nearly every tissue of the body, as well as to a number of "plastic" surfaces that are used to make dental protheses, catheters, and prosthetic cardiac valves (Kennedy, 1988). In this section, a detailed discussion of the physiochemical factors involved in C. albicans adhesion to various surfaces is presented, and a number of adhesion and association mechanisms are described. These are summarized in Table 2.

Physiochemical Considerations of Adhesion. The adhesion of C. albicans to any surface in vivo may vary considerably depending on host species, physiology, cell phenotype, and tissue involved (Anderson & Odds, 1985; Ryley, 1986; Segal et al., 1984; Sobel et al., 1981). Likewise, the attachment of C. albicans to exfoliated

Table 2. Adhesion and association mechanisms of *Candida albicans**

Mechanism	Nature of Mechanism		
	Active or Passive	Specific or Nonspecific	Direct or Indirect
Adhesin-receptor interaction	A	S	D,I
Nonspecific adhesion	A,P	N	D,I
Coadhesion to adherent organisms	A	S,N	I
Entrapment in mucus or tissue	P	N	D
Germtube penetration	A	N,S	D,I
Enzymatic digestion	A	N,S	D,I

*Modified from Kennedy (1988).

epithelial cells, tissue explants, or various nonbiological surfaces, such as plastic or glass, can vary greatly *in vitro* depending on a number of experimental factors (e.g. growth medium; see the discussion on adhesion models below). Moreover, a number of long range, short range and hydrodynamic forces, only a few of which have been studied, may influence *C. albicans* adhesion ability (Loeb, 1985; Marshall, 1976 & 1984; Rutter & Vincent, 1980 & 1984; Skerl *et al.*, 1984). Relatively little is known about the binding sites of *C. albicans* adhesins and even less is known about *Candida* binding kinetics. Because a complete physiochemical description of the adhesion of *C. albicans* to any one surface is not yet possible, the following discussion will present the fundamental physiochemical principles involved in *Candida*-substratum interactions.

In general, the cell surfaces to which *C. albicans* attaches, as well as the surfaces of *Candida* cells, have an overall negative surface charge (Jones, 1977). These negative potentials result from the ionization of various chemical groups (e.g., sialic acid carboxyl groups of epithelial glycocalyces, acidic amino acid side chains, glycolipids and phospholipids, etc.) of the cell surface. Likewise, glass and plastic surfaces may also possess a net negative surface charge, depending, of course, on the type and ionic strength of the surrounding milieu (Marshall, 1976). These charged surface groups loosely

attract oppositely charged ions (gegen- or counter-ions) from the surrounding solution to form a diffuse double layer of ions (Marshall, 1976). The resulting electric double layer is considered to be a part of the cell surface, in that as the cell is caused to move (e.g., due to Brownian motion or with an electric field) the thin layer of fluid and the ions of the double layer move with the cell (Jones, 1977).

Two models of the diffuse double layer have been proposed (Loeb, 1985; Marshall, 1976). One concept, the Stern model, assumes that there is a one molecule thick layer of counter-ions held at the surface (the Stern layer) by electrostatic and London-van der Waals attraction forces which are sufficiently strong to overcome thermal agitation. The other concept, the Gouy-Champman model, describes that of a diffuse double layer without the Stern layer. These concepts have been described in detail elsewhere (Loeb, 1985; Marshall, 1976) and will not be discussed further here. However, it should be noted that the ionic strength of the surrounding environment can greatly influence the dimensions of the dielectric layer, and, therefore, can also modify attractive and repulsive forces (Jones, 1977) because repulsion will occur when both layers overlap (Paerl, 1985). Not surprisingly, several studies have shown that C. albicans adhesion to epithelial cells varied according to selection of assay medium (Kimura & Pearsall, 1978 &

1980; McCourtie & Douglas, 1981; Persi et al., 1985).

The most useful descriptions of adhesive interactions between two negatively charged surfaces, which theoretically should repel one another, are those provided by the now classical lyophobic colloid theory of Derjaguin and Landau (1941) and of Verwey and Overbeek (1948) (DLVO theory) and its many derivations (Jones & Isaacson, 1983). Briefly, the DLVO theory proposes that the forces of repulsion (electrostatic interactions in the overlapping double layers) and attraction (London-van der Waals forces) between two similarly charged surfaces are additive, but vary independently with the distance of separation between the bodies. There are two separation distances between cells at which attraction is greater than repulsion. These are called the primary minimum (at small distances of separation of <1 nm) where attraction forces are strong, and the secondary minimum (at relatively large distances of separation of >10 nm) where attraction forces are weaker and are easily reversible, for example, by mild fluid shear (Jones & Isaacson, 1983). Interposed between these two positions is a point at which repulsive forces predominate and the potential energy is maximized (Jones & Isaacson, 1983). The overall charge and shape of the bodies are important and contribute significantly to the net interaction (Isaacson, 1985). For example, repulsion forces decrease with bodies of decreasing radius of curvature (Isaacson, 1985; Jones &

Isaacson, 1983). Likewise, as ionic strength in an environment increases the energy maximum repulsion barrier decreases, and at high electrolyte concentrations the repulsion energy barrier may be eliminated altogether (Jones & Isaacson, 1983).

The DLVO theory describes long-range adhesive interactions (i.e., those occurring at the secondary minimum) adequately where the bodies are held in a state of mutual attraction, but is inadequate in describing close-range interactions between adhesin and receptor, because bacteria and yeast do not possess sufficient kinetic energy to overcome the repulsion barrier (Jones & Isaacson, 1983; Rutter & Vincent, 1984). Nevertheless, the DLVO theory remains valid to describe certain adhesive interactions (Jones & Isaacson, 1983; Rutter & Vincent, 1980), and suggests that relatively irreversible adhesion (i.e., binding at the primary minimum) may require adhesive appendages (i.e. adhesins) that bridge the gap between cell surfaces to bind its complementary receptor (Jones & Isaacson, 1983, Marshall, 1984). Therefore, those interactions which occur at the secondary minimum are considered to be nonspecific and preliminary to irreversible adhesion (Jones, 1984a). Both types of interactions may be necessary for adhesion to and colonization of host tissues. For instance, although curved bodies come closer together at the secondary minimum and require less kinetic energy to reach the

primary minimum, and adhesion of yeast cells to cells with microvilli is energetically more favorable compared with other tissue of planar configuration, the effect of curvature may not decrease the repulsion sufficiently to permit C. albicans to reach the primary minimum.

Thus, in a system such as the small intestine, the effect of fluid shear due to peristalsis and the bathing actions of mucosal secretions should dislodge Candida cells resulting in their removal from the tissue surface. Therefore, as C. albicans is known to bind essentially irreversibly to small intestinal microvilli (Pope & Cole, 1981 & 1982), it is likely that specific adhesin-receptor binding is involved (Kennedy et al., 1987). However, without the weak attraction at the secondary minimum it seems unlikely that the presumed stereochemical fit of adhesin with receptor could take place (Fletcher, 1980; Jones, 1984a). Marrie and Costerton (1981) noted two types of Candida-epithelial cell interactions that preceded cell invasion. The first was a "loose" adhesion apparently mediated by a ruthenium red positive matrix, followed by a "tight" adhesion where no space could be seen between host and yeast cell.

Furthermore, it seems likely that adhesive appendages of very small radii relative to the yeast cell would favor contact with a tissue surface. Individual fibrillar adhesins, as those described earlier, with considerably smaller radii than the yeast cell could overcome repulsion

due to the potential energy maximum required to bridge the gap between C. albicans and epithelium. Lee and King (1983b) found that "fibrils" from C. albicans yeast phase cells mediated adhesion to vaginal epithelial cells and endothelial cells lining renal peritubular capillaries. It is also interesting to note that cells of C. albicans which possessed germtubes were found to be significantly more adhesive to BECs than were blastospores which did not possess germ tubes (Kimura & Pearsall, 1980; Sandin et al., 1982). It follows from the physiochemical principles described that the smaller radii of curvature of tiny germ tubes and smaller yeast cells may have facilitated adhesion. The alternative proposition that the increased adhesive activities for cells with germtubes resulted from a concentration of adhesins, or the production of additional adhesins, cannot be ruled out at present. Nevertheless, the smaller radii of curvature of germtubes are more energetically favorable for adhesion.

At short distances several other interactions are important in adhesion. Such interactions include: dipole-dipole (Keesom) interactions, dipole-induced dipole (Debye) interactions, ion-dipole interactions, chemical bonds (e.g., electrostatic, covalent and hydrogen), and hydrophobic interactions (Rutter & Vincent, 1980 & 1984; Tadros, 1980). A number of these types of interactions have been shown to be important in bacterial adhesion (Marshall, 1984). It should be noted, however, that

short-range effects may be repulsive or attractive depending on the nature of the surfaces involved (Rutter & Vincent, 1980; Tadros, 1980), and are particularly important in aqueous systems (Marshall, 1976; Rutter & Vincent, 1984). The role most of these short-range forces may play in the adhesion of C. albicans to biologic or nonbiologic surfaces has not been examined. Another possibility that also has not been considered is that the variability of the surface of C. albicans (e.g., in charge, shape, appendages, etc.) may lead to various types of synergistic interactions that take place simultaneously. In bacterial systems, hydrophobic interactions adjacent to ionic or hydrogen bonds have been shown to stabilize an otherwise energetically weak binding complex (Doyle et al., 1982). Synergistic interactions can easily be imagined for Candida adhesion and should be investigated. Likewise, the effect surface topography (Kulkarni et al., 1980) may have on Candida adhesion has been examined (Kennedy, 1988).

Only a limited number of physiochemical parameters have been studied in relation to C. albicans adhesion. Lee and King (1983b) examined the effect of certain physiochemical parameters on adhesion of C. albicans to human vaginal epithelial cells in vitro, and found that divalent cations, detergents, salts, and urea had no effect on adhesion. These findings suggest that hydrophobic, electrostatic or ion-bridging bonds may be of

little importance in adhesion to vaginal mucosal cells in this system. It should be emphasized that such results may be particular to the C. albicans strain and growth medium (and other assay parameters) used in that study. On the other hand, reducing agents (e.g., β-mercaptoethanol and dithiothreitol) significantly diminished C. albicans adhesion, but did not effect viability (Kimura & Pearsall, 1980; Lee & King, 1983b), suggesting that the structural integrity of the adhesive factor(s) is important.

In contrast to the study of Lee and King (1983b), Karaev et al. (1986) showed that the introduction of Ca^{2+} and Mg^{2+} ions to in vitro assays led to a significant increase in C. albicans adhesion to BECs. Likewise, divalent cations were shown to promote the adhesion of C. albicans to acrylic, and at high concentrations these cations caused extensive coadhesion and aggregation of C. albicans (McCourtie & Douglas, 1981). These studies indicate that ion-bridging mechanisms and electrostatic forces may be of primary importance in the adhesion of C. albicans to BECs, at least under some conditions, and acrylic surfaces in vitro.

Hydrophobic interactions also have been examined for their role in the adhesion of C. albicans to various denture base resin materials, plastics, and BECs (Klotz et al., 1985; Minagi et al., 1985 & 1986; Miyake et al., 1986). Klotz et al. (1985) found that the adhesion of C.

albicans to plastic surfaces was predominantly controlled by hydrophobic forces, and that electrostatic forces also contributed to adhesion. This view is corroborated by the findings of Minagi et al. (1985 & 1986), who showed that C. albicans could attach to denture materials in a similar manner. However, in the latter study C. albicans was significantly less hydrophobic, indicating that the surface free energy of the denture material itself greatly influenced Candida adhesion. Whereas hydrophobicity appeared to contribute to the adhesion of C. albicans to various plastics and denture materials, other nonspecific forces also probably contributed to the adhesion process. In contrast, recent studies from our laboratory showed that hydrophobicity may be of little importance in association with intestinal mucosa (Kennedy et al., 1987). However, hydrophobicity may have been important in yeast-to-yeast coadhesion, and may have indirectly influenced the total number of attached yeast by promoting coadhesion (Kennedy, 1988). The role cell surface hydrophobicity plays in C. albicans adhesion in vivo remains to be demonstrated.

Mucosal surfaces. The adhesion and association of C. albicans with oral, vaginal and GI mucosal surfaces has been studied in a number of in vitro and in vivo systems. Such studies have included adhesion to exfoliated epithelial cells, colonization and invasion of various

tissue explants, as well as the ultrastructural characterization of attachment and penetration of C. albicans to oral epithelium obtained from infected patients or experimental animals. Some of these studies have focused on the nature of the adhesion, whereas others have focused primarily on tissue localization.

Howlett and Squire (1980) examined the colonization and invasion of oral epithelium using tissue explants. Tissues were obtained from Sprague-Dawley rats and 4 day-old or younger New Zealand white rabbits, maintained in vitro in a chemically defined medium, and inoculated with C. albicans. Infected explants were maintained in vitro for 12 to 30 hours, and harvested at regular intervals to examine the various features of adhesion and association. Three types of interactions were noted. Blastoconidia of C. albicans were observed to be randomly adhering to the surface of the epithelium, and in many cases germtubes were observed extending from the parent blastospores and penetrating into the tissue. Adhesion of yeast cells to oral epithelium appeared to form an "intimate" contact between the cell surface and certain layers of the Candida cell wall. Five distinct layers in the cell wall of C. albicans could be observed in infected tissues, and the surface structure that appeared to mediate attachment was a floccular layer. Moreover, the cell wall of C. albicans appeared to undergo ultrastructural changes during adhesion. This is similar to the observations of Tronchin

et al. (1984) who noted ultrastructural modifications of the fungal cell wall coat during the adhesion of C. albicans to BECs in vitro. In that study, various cytochemical staining techniques were used to visualize the adhesion process, and showed that C albicans developed a fibrogranular surface layer that appeared to mediate adhesion. Concanavalin A binding sites were increased after development of this material (Tronchin et al., 1984).

The initiation of contact between Candida germtubes and the epithelium may represent a distinct adhesive mechanism because no alteration of the epithelial cell surface was noted either at the point of entry or around the vicinity of Candida microcolonies. This was true for all types of epithelia examined. Studies by Sandin and co-workers (1987a) suggested that germtubes may contain different adhesins, or concentrated amounts of adhesin (Sandin et al., 1982). Others have also reported that germinated yeast cells were significantly more adhesive than their non-germinated counterparts (Kimura & Pearsall, 1980). Within the epithelial cells of infected explants occasional loss of cytoplasmic components in the vicinity of the invading hyphae was noted (Howlett & Squier, 1980). These findings indicate that if enzymatic lysis was associated with the invasive process it was localized, probably to the hyphal tips. Mechanical support provided by the adhesion of Candida cells to the epithelium may

also facilitate growth and penetration into tissues (Howlett & Squier, 1980). Other reports support the role of both hydrolytic enzyme activity (Barrett-Bee et al., 1985; Ghannoum & Elteen, 1986; Pugh & Cawson, 1975, 1977 a&b, & 1978; Waid, 1978) and mechanical force (English, 1963) in the invasion of animal cells by this fungus. There was no evidence that non-germinated yeast cells invaded the epithelium directly. In addition to yeast adhesion and germtube penetration, it also was observed that long hyphae grew on and colonized the epithelial surface and penetrated deep into the tissue. Examination of the infected explants showed the hyphal form to predominate within the epithelium, especially at later times (Howlett, 1976). Sequential characterization of hyphal penetration of the rat tongue showed further that penetration occurred in three stages: (i) penetration of the superficial keratin layer (at approximately 18 hours), (ii) penetration of basal epithelium and basement membranes (at approximately 28 hours), and (3) invasion of connective tissues (at approximately 35 hours) (Howlett, 1976).

A number of studies examining infected tissues from patients and experimental animals have shown results similar to those described above. Montes and Wilborn (1968 & 1985) examined scrappings of plaque from the tongue of patients with chronic mucocutaneous candidosis and found pseudohyphae of C. albicans growing profusely

between epithelial cells and penetrating into them. In analogous studies, these authors found that Candida pseudohyphae grew on and colonized the surface of the buccal mucosa, and pseudohyphae penetrated keratinized cells (Wilborn & Montes, 1980). Pseudohyphal forms were predominant in patients with chronic mucocutaneous candidosis, although blastoconidia were often observed attached to and colonizing oral epithelium.

In another study (Marrie & Costerton, 1981), examination of plaques from the tongue and buccal mucosa of patients with oral candidosis revealed similar interactions between C. albicans and oral mucosa. These include a "loose" adhesion apparently mediated by a ruthenium red positive matrix (similar to floccular adhesins), a "tight" adhesion where no space could be seen between yeast and epithelium, and invasion of host cells by C. albicans hyphae. Coadhesion of Candida blastoconidia and indigenous bacteria to hyphal elements were also frequently noted (Marrie & Costerton, 1981). The adhesion of C. albicans to BECs in vitro was characterized by electron microscopy and appeared to proceed via similar mechanisms (Calderone et al., 1984). Again, yeast adhesion appeared to be of both a tight and loose nature. In addition, yeast cells were often observed in phagocytic vacuoles of epithelial cells. From these and similar studies it is not clear whether these two ultrastructural forms represent distinct adhesive

activities or phases of the same process. Although tight and loose adhesion of C. albicans could represent two different mechanisms of adhesion, they probably represent different stages of the same adhesion mechanism (Sandin, 1987b) similar to phases of reversible adhesion that precede irreversible adhesion in bacteria (Jones & Richardson, 1981 a&b). Studies using 0.4% formalin saline to kill Candida cells revealed that dead organisms formed a loose adhesion but that only viable cells bind irreversibly (Vudhichaminog et al., 1982).

As noted earlier, a number of studies have tried to characterize the nature of Candida adhesin(s), and to a much lesser extent epithelial cell receptor(s), by introducing various substances into an assay that might block adhesion or by pre-treating host or yeast cells before preforming adhesion assays. Using the former approach it was found that C. albicans may bind reversibly for as long as 20 minutes, but thereafter the cells bind irreversibly (Sandin, 1987b). This suggests that the initial yeast-epithelium contact may be due to non-specific adhesion, followed by specific adhesion, or that, if the initial attachment is the result of specific adhesion, for example, "loose" binding via floccular adhesins, additional adhesion or association mechanisms may be required for irreversible binding (Sandin, 1987b). Modification of both host and yeast cells have been observed once C. albicans attaches (Howlett & Squier,

1980; Tronchin et al., 1984). It may be that as Candida cells "bump" into epithelial cells and bind reversibly, physiologic changes occur to both host and fungus that strengthen the adhesion. These changes could modify the epithelium to the extent that more or different receptors are exposed that could stabilize and strengthen adhesion. Likewise, modifications of the cell wall of C. albicans, possibly causing an increase or concentration of adhesins, or an unmasking of different or more adhesins, have been noted at yeast-epithelial binding sites.

Alternatively, the deposition of new cell wall material could occur after initial attachment. Tronchin et al. (1984) have noted the reorganization and proliferation of an external cell wall layer of C. albicans during adhesion to BECs. Moreover, they noted an abundant extracellular material with numerous binding sites for Concanavalin A that appeared to be released from the yeast surface, leaving underlying cell wall layers exposed. Several descriptions of phases in Candida adhesion to epithelial cells exist (Ghannoum et al., 1986a), but further studies to characterize the affinity and number of binding sites will be necessary to present a more complete and accurate description.

It also has been suggested that the adhesion of C. albicans to buccal mucosal cells might entail ionic interactions involving divalent cations (Karaev et al., 1986). The adsorption of macromolecules to epithelial

cells is recognized to occur via electrostatic interactions involving calcium ions and other ionic groups. Candida cells could also attach by similar mechanisms. However, conflicting data do not permit definite conclusions to be drawn. For example, it was shown that at acidic and basic pHs Candida adhesion decreases (Sobel et al., 1981). In another study (Samaranayake & McFarlane, 1982a), the opposite results were observed. These results, however, may be strain dependent (Persi et al., 1985). Also, cell surface hydrophobicity has been implicated in mediating Candida adhesion (Hazen et al., 1986). One may speculate, therefore, that once long-range electrostatic forces are overcome, specific adhesin-receptor interactions become the important determinants of adhesion.

Because of the possible ecological and pathological consequences of mucosal association by Candida in the GI tract (Eras et al., 1972; Krause et al., 1969), several methods have been used to examine the adhesion and association of C. albicans with gut mucosa. Most have involved removing infected tissues from animals and viewing the resulting association by scanning (SEM) or transmission (TEM) electron microscopy (Balish et al., 1984; Field et al., 1981; Kennedy & Volz, 1985a; Kennedy et al., 1987; Pope et al., 1979; Pope & Cole, 1981 & 1982). Others have included examination of stained histologic sections or quantitative cultures to determine

population levels of attached yeast in infected tissue (Pope & Cole, 1981 & 1982). Tissue slices or isolated mucus gel have also been used to study mucosal association *in vitro* (Kennedy & Volz, 1985a; Kennedy *et al.*, 1987; Segal *et al.*, 1986).

Animal models used to study colonization of the GI tract have included neonatal and adult conventional, specific pathogen-free, germ-free, antibiotic-treated, and/or athymic gnotobiotic mice, hamsters, chickens and rats (Guentzel *et al.*, 1985), and have revealed mucosal association by *C. albicans* in all regions of the GI tract (Balish *et al.*, 1984; Field *et al.*, 1981; Kennedy *et al.*, 1987). Different preferential sites of colonization, however, have been noted between various animal models. Pope *et al.* (Field *et al.*, 1981; Pope & Cole, 1981 & 1982), for instance, found that the stomach was the primary site of colonization in infant mice, whereas studies in antibiotic-treated adult mice have revealed that the cecum was most heavily colonized by *C. albicans* (Kennedy *et al.*, 1987; Turner *et al.*, 1976). It is also interesting to note that the GI tract of neonatal mice was not colonized as quickly with *C. albicans* as were adult mice (Balish *et al.*, 1984). Nevertheless, the aforementioned studies have demonstrated that *C. albicans* can associate with GI mucosa presumably by several different (distinct) mechanisms.

Examination of the stomach mucosa of infant mice

revealed that at early times after Candida inoculation, yeast cells were attached to both keratinized squamous epithelia and columnar secreting epithelial surfaces. At later times, yeast were also seen attached to the secreting epithelium surrounded by and embedded in mucus, and were observed to be associated with and attached to lactobacilli at the junction of the keratinized and secreting epithelium (Balish et al., 1984; Pope & Cole, 1981 & 1982). Hyphal invasion of the keratinized region of the stomach has also been observed in both infant and adult mice (Balish et al., 1984; Pope & Cole, 1981 & 1982). The cardial-atrium section of the stomach appears to be the preferential location in the stomach in some experimental animals (Guentzel et al., 1985), and in some studies it was observed to be the sole colonization and invasion site by C. albicans (Balish et al., 1984; Helstrom & Balish, 1979; Pope & Cole, 1981 & 1982). It may be that the cardial-atrium ridge contains an abundant number of receptors, which allow C. albicans to colonize this region of the stomach preferentially. These findings suggest that C. albicans can associate with stomach mucosa by a number of adhesion and association mechanisms. These include direct adhesion to the epithelium, indirect attachment to the epithelium by association with other microorganisms, and penetration of the epithelium. At present, no attempt has been made to identify Candida adhesins or mucosal receptors that might be involved in

gastric colonization by C. albicans.

The association of Candida with small intestinal mucosal surfaces has also been studied in infected animals. These studies have demonstrated that C. albicans can associate with intestinal mucosa by several different and distinct mechanisms (Field et al., 1981; Pope & Cole, 1981 & 1982). Using an infant mouse model, Pope and Cole (Field et al., 1981; Pope & Cole, 1981 & 1982) reported that large numbers of C. albicans were clearly visible on the surface of villi in the small intestine within a short time after inculation. Further examination revealed that yeast adhered to all areas of the epithelium. Many yeasts were also seen on the surface of the villi, frequently in association with mucus, and appeared to be attached to, embedded in, and covered by a layer of mucus. Histological sections at 3 and 6 hours post-challenge also revealed cells embedded in the villus surface, and that some yeasts were associating indirectly with the mucosa by attaching to adherent yeast. Ultrathin sections from small intestinal mucosa showed that yeast cells did not attach to microvilli, but instead they attached to overlying mucus.

Examination of large intestinal mucosal surfaces from adult animals revealed that C. albicans associated with these surfaces by similar mechanisms (Kennedy & Volz, 1985a; Kennedy, 1987). However, this was true only when the animals were given antibiotics to disrupt the ecology

of the indigenous bacterial flora (Kennedy & Volz, 1983 & 1985b). SEM studies revealed that large numbers of Candida cells were present on the surface of the epithelium and mucus in antibiotic-treated animals challenged with C. albicans, whereas no yeast cells were observed associating with host mucosal surfaces of untreated animals similarly challenged. In the latter case, large numbers of indigenous bacteria were seen colonizing the mucosa. In mice given antibiotics, C. albicans could associate with the mucosa of all areas of the GI tract, but the cecum had the highest population levels.

Further examination of the cecal mucosa of antibiotic-treated animals challenged with C. albicans revealed that Candida cells could attach to the epithelium, possibly by adhesion to the epithelial glycocalyx (Kennedy et al., 1987). In addition, yeast cells were observed to attach directly to or were seen embedded in mucus material. It was also found that C. albicans could associate with the cecal mucosal surface indirectly by attaching to other adherent organisms such as adherent yeast cells and bacteria.

The possible importance of these interactions becomes apparent in the observation that microcolonies of Candida have been observed in the cecum at 72 hours after oral challenge; with some yeasts associated with the epithelium, and others attached to mucus material and

other yeast cells (Kennedy et al., 1987). If these microcolonies are formed in intestinal mucosa in humans, this may explain the isolated plaques observed by certain workers on examination of intestinal mucosa infected with C. albicans (Eras et al., 1972; Joshi et al., 1981). Microcolony formation has been suggested to be of ecologic and pathologic importance in colonization of mucosal surfaces by other pathogens (Cheng et al., 1981; Costerton et al., 1981 & 1985). Depressions in the epithelium also were observed under Candida cells, possibly due to enzymatic lysis which may expose underlying receptors and stabilize C. albicans to the epithelium after the initial adhesion. It is interesting to note that germtubes or hyphae were not observed to penetrate the mucosa of the small or large intestine in these studies (Kennedy & Volz, 1985a, Kennedy et al., 1987).

Although C. albicans was observed to associate with, and appeared to attach to mucus in the specimens processed for electron microscopy described above, the mucus gel was not maintained intact and appeared to be somewhat amorphous. Therefore, to determine more conclusively if C. albicans could associate with the mucus gel proper, and to insure that the apparent attachment to mucus was not due to specimen processing (which can cause mucus to shrink), Candida adhesion to mucus gel was tested in vitro (Kennedy et al., 1987). It was found that large numbers of C. albicans associated with intestinal mucus very

rapidly (within 5 minutes) when mixed together in vitro, and that Candida organisms were attached to and embedded in the gel. Quantitative cultures of Candida in the mucosa of infected animals showed that approximately 20% of the associated C. albicans were present in the mucus gel (Kennedy et al., 1987).

The nature of the adhesive events between C. albicans and the intestinal mucosa, as well as between Candida and other adherent microorganisms, remains to be determined. It seems unlikely that nonspecific interactions, such as cell surface hydrophobicity, played a significant role in the adhesion of Candida to mucosa (Kennedy, 1987; Kennedy et al., 1987). Studies with bacteria are consistent with this view, and suggest that specific adhesion predominates over nonspecific adhesion (Jones, 1984b). Therefore, it seems likely that a complex adhesive system is involved in the attachment of C. albicans to various mucosal surfaces and other microorganisms, and may be mediated by two or more distinct adhesive entities in which nonspecific interactions (e.g., hydrophobicity) may or may not play a role (Kennedy, 1987; Kennedy et al., 1987).

Nonbiologic Surfaces. The adhesion and association of C. albicans with a number of nonbiologic surfaces can create a wide range of health problems for humans. Studies on the oral distribution of Candida in chronic atrophic candidosis, a disease that is relatively common among elderly denture wearers (Odds, 1988), have shown that C.

albicans is recovered more often and in higher numbers from the acrylic denture surface than from the palate (Davenport, 1970). This suggests that colonization of the acrylic denture surface serves as a reservoir for infection, and that Candida adhesion to the denture surface may be prerequisite for colonization and infection of the palate (McCourtie & Douglas, 1981). Likewise, the adhesion of Candida cells to various plastic surfaces that are used to make catheters and prosthetic cardiac valves, may also cause serious medical complications in a variety of patients. Relatively few studies on the adhesion of C. albicans to these surfaces, however, have been conducted.

The adhesion of Candida to acrylic has involved the majority of the studies (McCourtie & Douglas, 1981; McCourtie et al., 1985; Miyake et al., 1985; Samaranayake & McFarlane, 1980; Samaranayake et al., 1980), which have suggested that several factors are likely to be important in this association. Studies by McCourtie and Douglas (McCourtie & Douglas, 1981) have shown that growth of C. albicans in a chemically defined medium supplemented with a variety of carbohydrates can greatly modify adhesion to acrylic surfaces. Growth of Candida in medium supplemented with 500 mM galactose, for instance, showed a significant increase in the amount and rate of adhesion and the production of an extra floccular surface layer (McCourtie & Douglas, 1981). Production of this material, which may be a mannoprotein (McCourtie & Douglas, 1985a),

has also been found to cause an increase in adhesion to BECs (Douglas *et al.*, 1981) and an increase in virulence in a systemic mouse model (McCourtie & Douglas, 1984). These results have prompted some to suggest that dietary factors may have a profound influence on *Candida* adhesion *in vivo* (Samaranayake & McFarlane, 1982b & 1985).

Nevertheless, studies aimed at characterizing the adhesive nature involved in the attachment of *C. albicans* to acrylic surfaces and denture base resins have suggested that cell surface hydrophobicity may mediate, or at least participate in adhesion (Klotz *et al.*, 1985; Minagi *et al.*, 1986 & 1986; Miyake *et al.*, 1986). Miyake *et al.* (1986) found a strong correlation existed between the adhesion capacities of *Candida* species to acrylic surfaces and their cell surface hydrophobicities. It is interesting to note that in these studies *C. albicans* showed the lowest hydrophobicity (Minagi *et al.*, 1986; Miyake *et al.*, 1986), which was probably due to the growth medium and environmental parameters used (Kennedy, 1988). Growth of *C. albicans* in Sabouraud dextrose broth at 37°C produced cells that were only slightly hydrophobic (Miyake *et al.*, 1986). Therefore, it would be of interest to examine cell surface hydrophobicity and adhesion to acrylic after growth of *C. albicans* in a variety of media (Kennedy, 1988).

It is worth noting that four different methods have been used to determine the cell surface hydrophobicity of

C. albicans. These included contact angle measurements, separation in water-hydrocarbon two-phase systems, plastic adhesion, and hydrophobic interaction chromatography (Klotz et al., 1985; Miniagi et al., 1985 & 1986; Miyake, 1986; Reinhart et al., 1985). Comparison of the former two assays indicates that these methods gave similar results. Comparisons between hydrophobic interaction chromatography and the other two methods have not been conducted. All of these methods have certain drawbacks that may influence results. The currently described hydrophobic interaction chromatography method (Smyth et al., 1978), for instance, has the drawback that Candida cells may be occluded in the gel, especially if they occur as pseudohyphae or aggregates. A modification of this procedure, where the sample is allowed to mix with the gel instead of passing through it, has been used to avoid this problem (Kennedy, unpublished data).

Ion-bridging bonds have also been suggested to play a role in the adhesion of C. albicans to acrylic (McCourtie & Douglas, 1981). McCourtie and Douglas (1981) found that the addition of divalent cations to assay mixtures caused an increase in Candida adhesion to acrylic. The addition of Ca^{2+} was shown to cause a greater increase in adhesion, than was the addition of Mn^{2+} or Mg^{2+}. Cations seemed to cause a slight decrease in adhesion, and EDTA had no effect on its own but abolished the adhesion promoting effect of Ca^{2+}. Cells grown in medium containing glucose,

galactose, or sucrose were all found to have an increased adhesion due to Ca^{2+}, but this effect appeared to be greater with the latter sugar. $FeCl_2$ also promoted adhesion to acrylic, but at high concentrations it caused the yeast cells to aggregate and settle to the bottom of the assay wells (McCourtie & Douglas, 1981). Thus ion-bridging mechanisms, in addition to cell surface hydrophobicity and other unknown factors (Rotrosen et al., 1986), may play a role in the adhesion of C. albicans to acrylic surfaces.

Other nonbiologic surfaces have been studied less extensively than acrylic in regard to Candida adhesion. In one study, Candida species were shown to attach to both polyvinyl chloride and Teflon catheters, although yeasts adhered more extensively to the former (Rotrosen et al., 1983). Furthermore, it was also found that C. tropicalis adhered in greater numbers than C. albicans. In another study, the adhesion of Candida species was examined microscopically and revealed adherent Candida enmeshed in fibrin-like strands (Locci et al., 1981). Reinhart et al. (1985) studied the adhesion of C. albicans to glass and concluded that adhesion was not due to cell surface hydrophobicity. Factors that may govern adhesion of Candida to a variety of plastic surfaces were investigated by Klotz et al. (1985). These authors found that the adhesion of C. albicans to plastic was governed predominantely by hydrophobic properties of both the yeast

and plastic surface. Kinetic analyses of the interaction between C. albicans and plastic also revealed negative cooperativity determined by Scatchard and Hill plots, due to electrostatic repulsion. This was determined by altering the surface charge of Candida cells by selectively blocking amino and carboxyl groups or increasing the ionic strength of the assay solution, and conducting adhesion assays. The more positively charged yeasts adhered in greater numbers, suggesting that the more negatively charged cells were more repulsive to the negatively charged plastic surface. Electrostatic forces were, therefore, deemed to be minor compared to the hydrophobic forces, as the adhesion of C. albicans to plastic without theses pretreatments occurred at high frequency.

The investigations of the adhesion of C. albicans to acrylic and various plastics described above have provided some information that could be useful to prevent or reduce the adhesion of yeasts to these surfaces in vivo. However, further studies will need to be carried out to determine whether or not these in vitro phenomena are applicable to the situation in vivo. In the latter situation, numerous substances are adsorbed to these materials that probably alter their surface properties, and, therefore, the adhesion of yeast and bacteria to them. Saliva, which contains numerous components that adsorb to plastics and acrylic, has been shown to reduce

Candida adhesion to acrylic when it was applied to acrylic before use in adhesion assays (McCourtie & Douglas, 1981; Miyake et al., 1986a; Samaranayake et al., 1980). The adsorption of these components may also suggest that specific adhesin-receptor interactions are involved in adhesion to molecules adsorbed to plastics in host tissue. Likewise, all of the previously described experiments have used different growth media and culture conditions, which have been shown to influence Candida adhesion and hydrophobicity in vitro (Hazen et al., 1986). Although it is likely that these properties are important in the adhesion of C. albicans to various nonbiological surfaces, further studies are needed to determine the relevance of each in vivo.

Microbial ecology of gastrointestinal ecosystems

Because the succession, development and composition of the microbial flora of the GI tract has been the subject of several excellent reviews and books (Drasar & Hill, 1974; Finegold et al., 1983 a&b; Freter, 1983 & 1984; Moore, 1977; Savage, 1977 & 1980), a complete overview of these topics will not be possible here. However, before a discussion on the factors or bacteria that control C. albicans populations in the gut can be made, it will be necessary to at least list the major features of the GI microflora. These include: the complexity of the GI microflora, the diversity of the

microbial species found in various niches of the GI tract, and factors that might influence the composition of the microflora. By beginning with this subject, it is hoped that an appreciation can be gained for some of the stringent details that are involved with, and which can influence, the study of the survival, dissemination, and pathogenesis of C. albicans and other fungi in the GI tract. The following paragraphs were modeled after an excellent review by Freter (1983) and were supplemented to include the following generalizations:

1. It is generally believed that the intraluminal contents of the normal human stomach is relatively sterile, with only low numbers ($\leq 10^3$ colonies per ml gastric contents) of a variety of organisms being present (Finegold et al., 1983b; Franklin & Skoryna, 1966; Giannella et al., 1972). Studies indicate that these low counts may well represent oral and ingested microorganisms since counts tend to decrease to undetectable levels several hours after eating (Draser et al., 1969; Franklin & Skoryna, 1966), which is most likely due to changes in gastric pH (Giannella et al., 1972). Despite the apparent harshness of the stomach to colonization by microorganisms, some investigators have noted that certain microorganisms can be seen attached to the epithelium and deep in foveae in histologic preparations viewed by light or electron microscopy (Savage, 1970). A similar descripton of the stomach flora of laboratory rodents has

also been detailed, but the microbial species associated with the gastric mucosa are very different (Savage, 1969 & 1980). It should be noted that the indigenous gut microflora, for any portion of the GI tract, probably differs from host to host, even within the same species (Moore & Holdeman, 1974). Studies have shown, however, that α-hemolytic streptococci, anaerobic cocci, lactobacilli, Staphylococcus epidermidis, and C. albicans make up the vast majority of microorganisms that colonize the stomach (Franklin & Skoryna, 1966; Giannella et al., 1972). The microflora of the small intestine in the normal individual or laboratory animal, in contrast, contains numerous bacteria that are highly dependent on the location of sampling. Generally, the microbial counts range from being sterile (or very low) to very high as samples are taken from the duodenum and descending to the ileum, respectively (Finegold et al., 1983b). Several studies have revealed that in the upper small intestine of normal healthy human subjects, low counts ($0-10^{4.5}$ colonies per ml) of both aerobic (streptococci, staphylococci, lactobacilli, and yeasts) and anaerobic (streptococci and lactobacilli) microorganisms, and almost a complete absence of coliforms and Bacteroides species (Finegold et al., 1983). As samples are taken farther along the small intestine, at the distal ileum, mean bacterial counts increase significantly ($10^{3.5}$ - $10^{6.5}$ colonies per ml) and the microflora more closely resembled

colonic flora with higher counts of coliforms and Bacteroides species (Finegold et al., 1983b; Plaut et al., 1967). The large intestinal flora of the healthy host consists of an average of at least 10^{11} colonies per gram which contains some 400-500 (mostly strictly oxygen-sensitive anaerobes) separate bacterial species from more than 45 genera (Draser & Hill, 1974; Holdeman et al., 1974; Moore, 1977; Savage, 1977). Finegold et al. (1983b), in their review of the "normal" indigenous microflora, have analyzed and summarized over 10 years of indepth studies characterizing the colonic microflora from several hundred individuals with various disease states and who were on various diets. These authors noted that there was a remarkable similarity between the major bacterial groups in general despite major differences in diet, disease state, and nationality. Table 3 summarizes the mean bacterial counts and percentages of specimens positive for the major bacterial groups present in the fecal flora as described in these studies. Several factors that may alter the composition or metabolic activity of the microbial flora of the gut include stress, diet, prolonged antibiotic and immunosuppressive therapy, surgery, the individuals genetic background, and a number of infections and debilitating diseases. All of these have been commented on elsewhere (Caugant et al., 1981; Draser et al., 1969; Freter, 1976; Gerhardt & Iglewski, 1976; Gracey et al., 1974; Hartley et al., 1978; Moore,

Table 3. Major bacterial groups present in human fecal flora*

Bacterial group	% of total specimens positive	Log_{10} number of organisms per gram (dry weight)	
		Mean	Range
Actinomyces	84.2	9.2	3.8 - 11.1
Anaerobic cocci	94	10.5	4.1 - 13.4
Arachmia-Propionobacterium	9	9.0	4.3 - 12.0
Bacteroides	99	11.3	9.5 - 13.8
Bifidobacterium	74	10.2	5.0 - 13.6
Clostridium	100	9.8	3.8 - 13.1
Eubacterium	94	10.7	5.1 - 13.5
Fusobacterium	18	8.5	5.1 - 11.0
Gram-negative facultative anaerobes	98	8.5	4.0 - 10.0
Other facultative anaerobes	93	6.5	1.0 - 11.2
Lactobacillus	78	9.5	3.6 - 12.3
Streptococcus	99	8.9	4.0 - 12.8

*Adapted from Finegold et al. (1983b).

1977; Moore et al., 1981; Moore & Holdeman, 1974; Savage, 1977), but several of these may predispose individuals to colonization and infection with Candida (Odds, 1988).

2. The indigenous microflora of a given location within the GI tract, including various microsites within that location, is stable in its composition because the GI tract is an ecosystem in the climax stage (Zubrzycki & Spaulding, 1957 & 1962). Thus "invading" microorganisms are usually prevented from colonizing the gut, unless, of course, they enter this site before the indigenous microflora has become established or after the microflora has somehow been disrupted (Dubos et al., 1963; Finegold et al., 1983a; Freter, 1983). This phenomenon has been described using several terms (e.g., bacterial antagonism [Freter, 1956], bacterial interference [Dubos, 1963] and colonization resistance [van der Waaij et al., 1971]), and constitutes a potent host defense mechanism (Freter, 1983). However, as noted by Freter (1983), although the gut microflora is stable in its function, the stability of individual bacterial species is not absolute. Consistent with this is the finding that the composition of the colonic microflora varies considerably in different hosts (Moore, 1977; Moore et al., 1981; Moore & Holdeman, 1974). Furthermore, it has been noted that, due to unknown factors, the population size of some indigenous bacterial species apparently may change from day to day within the same individual (Moore, 1977; Moore et al., 1981; Moore &

Holdeman, 1974). One relevant example, as has been noted by Freter (1983), is that some individuals harbor no recognizable "resident" strains of Escherichia coli but only a frequent succession of different "invader" or "transient" E. coli strains (Anderson et al., 1973; Caugant et al., 1981; Gerhardt & Iglewski, 1976; Sears & Brownlee, 1952; Sears et al., 1949; van der Waaij et al., 1971), and there are occasional individuals who allow "invader" strains to transiently coexist with "resident" strains (Anderson et al., 1973; Caugant et al., 1981; Gerhardt & Iglewski, 1976; Sears & Brownlee, 1952; Sears et al., 1949; van der Waaij et al., 1971). A similar situation may exist for C. albicans (Abdelghaffar & Russell, 1979), but no definiative studies have been conducted to follow up this isolated observation. Nevertheless, one major feature of the gut microflora is its ability to resist implantation by allochtonous microbial species.

3. There are two general habitats of colonization in GI ecosytems, namely the lumen contents and the mucosa (Freter, 1983; Savage, 1977). This has been observed in several animal species and in man, and it has been found that both of these sites may contain common and/or unique microbial species (Freter, 1982 & 1983; Savage, 1970 & 1980). Furthermore, within the mucosa there may be two micro-sites, which include those cells directly attached to the epithelium and cells entrapped in what is usually

described as mucus (Freter, 1982 & 1983; Kennedy et al., 1987; Savage, 1983). Recent studies indicate further that these micro-sites are closely interrelated in that a primary layer of bacteria "attaches" to the epithelium, and that several subsequent "layers" adhere to the primary one and to each other (Freter, 1983). As will be noted below, for an area of the GI tract to function properly, at least in terms of resisting colonization to Candida and other invaders, the mucosa-associated flora must remain intact.

Importance of gastrointestinal colonization by Candida albicans

Colonization of the GI tract by Candida species, and C. albicans in particular, may play an important role in human health and disease since the GI tract has been implicated as an important source of infection in cases of napkin dermatitis (Rebora & Leyden, 1981), recurrent vaginitis (Miles et al., 1977; Nystatin Multicenter Study Group, 1986) and systemic candidosis (Krause et al., 1969; Myerowitz et al., 1977; Stone, 1974; Stone et al., 1973 & 1974; Walsh & Merz, 1986). The over-growth of C. albicans in the intestinal tract, and its subsequent passage through the gut mucosa into the host bloodstream, for instance, is believed to be the proximate mechanism leading to systemic candidosis (Stone et al., 1974), particularly in patients with acute leukemia (Meunier-

Carpenter et al., 1981; Myerowitz et al., 1977; Ray, 1980). Similarly, colonization of the GI tract by C. albicans and other Candida species may also lead to involvement in a number of other disease syndromes that include esophageal, gastric and intestinal thrush, gastric and intestinal ulceration, GI bleeding, diarrhea, peritonitis, perianal itch, napkin dermatitis, chronic "irritable bowel" syndrome, and auto-brewery syndrome (Odds, 1988). The importance of GI colonization by C. albicans, therefore, should not be underestimated, especially in view of the suggestion that "the most important source of Candida species in human disease is endogenous" (Odds, 1988). On the other hand, GI colonization by C. albicans may also have important beneficial effects on the host, in that it apparently results in a stimulation of the immune system leading to a protective response to systemic Candida infection in the noncompromised host (Domer, 1988; Domer & Hector, 1987).

The clinical recognition of candidosis of the GI tract has been traced by Odds (1988) to the late 1700's, which was long before C. albicans was discovered (Odds, 1988; Rippon, 1988). Nearly a century elapsed from this date, when C. albicans was first isolated from a patient with candidosis of the stomach and colon (Odds, 1988; Rippon, 1988). The modern revival of the importance of GI tract colonization colonization by C. albicans, however, had to wait nearly another century, and can probably be

credited, at least in part, to Krause and co-workers (1969) who demonstrated the rapid "persorption" of C. albicans through the GI mucosa into the bloodstream when Krause drank a saline suspension containing 10^{10} viable C. albicans. This was augmented by a number of studies that examined the role of the "normal" intestinal flora in inhibition of C. albicans in experimental animals (Balish et al., 1974; Balish & Phillips, 1966 a&b; Clark, 1971; Hazen et al., 1953; Huppert et al., 1955; Isenberg et al., 1960; Paine, 1958; Phillips & Balish, 1966) and to several clinical studies of the occurrence of Candida gut colonization in various patient populations (Dewilde et al., 1982; Stone, 1974; Stone et al., 1973 & 1974). Widespread acceptance of this idea, however, had to wait a few more years until Myerowitz et al. (1977) showed a correlation between GI colonization by C. albicans and systemic infection in leukemic patients and to Miles et al. (1977) who showed a similar relationship to recurrent vaginitis. The latter findings, especially together with more recent studies (Balish et al., 1984; Ekenna & Sheretz, 1987; Field et al., 1981; Hector & Domer, 1982 & 1983; Kennedy & Volz, 1983 & 1985 a&b; Kennedy et al., 1987; Lee & Balish, 1982; Pope et al., 1979; Umenai, 1978; Umenai et al., 1979 & 1980; Wingard et al., 1980 & 1982), has now brought new prominence to the idea that colonization of the GI tract by C. albicans may serve as an important reservoir for both superficial and systemic

candidosis (Odds, 1988). Recent data obtained from experimental animals reinforce this view and have shown that a number of fungi can spread systemically from the GI tract (Kennedy & Volz, 1983).

Odds (1988) in his review on "Candida and candidosis" has examined in detail clinical and pathological features of GI candidosis, and has painstakingly summarized the incidence of GI carriage in "normal" and "patient" populations. Therefore, these topics will not be considered here. Nevertheless, two significant facts that are evident from this work should be noted before beginning a discussion on the survival and implantation of Candida in the GI tract. (i) Not all healthy individuals, regardless of their age, sex, or the number of attempts (or methods) used to isolate "yeasts" from the GI tract, harbour a "resident" yeast flora. According to Odds (1988), about 40 to 50% of a given sample population can be shown to carry Candida throughout most of the length of the GI tract. The incidence of Candida carriage, furthermore, has been shown to be highest in the oral cavity and lowest in the colon (Odds, 1988). Because C. albicans can pass through the GI tract apparently without being "killed" (Kennedy & Volz, 1985a), the percentage of healthy individuals harbouring C. albicans in their gut is probably lower; since some positive fecal cultures may represent Candida removed from the oral cavity. Thus, common statements like "all of us acquire

Candida at birth", or "all of us carry Candida as a part of our normal flora", are unfounded. (ii) The incidence and population size of C. albicans in the gut, nonetheless, usually increases in individuals who are predisposed by illness, debility or a local reduction in host defense mechanisms (Odds, 1988; Rippon, 1988). Table 4 summarizes factors thought to remove, or at least lower, the "barrier" that inhibits the overgrowth of C. albicans in the GI tract. Table 5 summarizes some recent data on the carriage of Candida species in the gut as related to diet.

It is apparent from these brief comments that an overgrowth of C. albicans in the GI tract, and its subsequent pathologic consequences, could arise in a compromised individual either because of already harbouring a resident C. albicans population or by acquiring a "hospital strain" because the barrier to implantation was reduced or eliminated altogether. There is good evidence for both conditions (Abdelghaffar & Russell, 1979; Carpenter, 1955; Cohen et al., 1969; Kennedy & Volz, 1985a; Pope et al., 1979; Shepherd et al., 1985) and it is not yet clear which is more relevant than the other. Nevertheless, both positions underscore that C. albicans can become implanted in the gut either before the normal microflora has become established (e.g., during infancy) or after the ecological balance of the microflora has been disrupted (e.g., by antibiotic treatment). The

Table 4. General classification of factors thought to predispose to colonization and dissemination from the gastrointestinal tract by *Candida**

Classification of predisposing factors	Explanation	Examples
Natural	1. Infectious, idiopathic, congenital or other debilitating diseases and disorders	Microbial infections, endocrine dysfunctions, defects in immunity
	2. Digressions from normal hormonal or physiological status	Pregnancy, infancy
Dietary	1. Excess or deficiency of foodstuffs that may alter the composition of the microbiota or the metabolic activity of the flora	Carbohydrate-rich diets, vitamin deficiencies
	2. Intake of foodstuffs that may alter local immunity	
Mechanical	1. Trauma	Stabbing
	2. Prolonged stay in a closed environment	Space flight
Iatrogenic medical	1. Treatment with drugs that alter the composition of the microbiota or suppress host defense mechanims	Antibiotics, corticosteroids
	2. Surgical procedures	Bowel resections

*Adapted and modified from Odds (1988).

Table 5. Isolation of Candida albicans and other yeasts in stool samples from healthy individuals on different diets*

Diet	Organism	% of total samples positive	Log_{10} numbers of organisms per gram	
			Mean	Range
Japanese	C. albicans	47	5.6	3.5 - 8.9
	Candida spp.	13	6.3	3.7 - 8.8
	Other yeasts	53	5.8	3.4 - 8.7
Strict vegetarian	C. albicans	15	4.9	3.5 - 6.3
	Candida spp.	0	---	-------
	Other yeasts	23	5.6	4.3 - 7.8
Vegetarian some meat	C. albicans	0	---	-------
	Candida spp.	0	---	-------
	Other yeasts	50	6.1	4.2 - 8.7
Western	C. albicans	14	5.4	3.6 - 9.4
	Candida spp.	8	4.4	3.7 - 5.1
	Other yeasts	31	5.2	3.6 - 8.1

*Data from Finegold et al. (1983b).

survival and implantation of Candida in the gut will, therefore, be considered from both views. Unfortunately, there is no study that has followed a large group of human subjects from birth to adult, for the specific purpose of monitering yeast flora population dynamics and/or the acquisition of new yeast strains. Therefore, the comments below will be derived almost entirely from data obtained from experimental animals.

A number of animal models of GI colonization and dissemination by C. albicans have been developed and used for studies of antifungal chemotherapy, colonization, mucosal association and invasion, and pathogenesis. These models have included several animal strains and species, and have included the use of both adult and neonatal conventional, specific pathogen-free, germfree, gnotobiotic, and athymic germfree or gnotobiotic animals (Guentzel et al., 1985). For obvious reasons, laboratory rodents, particularly mice, have been the most extensively used models described to date. No attempt will be made here to describe any of these models in any particular detail, but the interested reader is referred to the review of Guentzel et al. (1985) who have described in some detail most of the currently available animal models for studying GI colonization and dissemination by C. albicans. Certain salient features unique to a particular model that may aid in the identification and/or elucidation of the determinants of gut colonization or

invasion of the intestinal mucosa by C. albicans, however, will be noted where appropriate. The following discussion focuses on the ability of C. albicans to survive and implant in the gut and examines the effects various pre- or post-treatments have on colonization and systemic spread of Candida from the GI tract.

Several studies have been conducted over the past 35 years that make it impossible in the space given here to completely describe and compare the numerous data that has been generated on gut colonization by Candida. Therefore, it became apparent at this writing that the only way to make such a comparison, especially when considering the wide variation in experimental parameters that has been used between laboratories, was to construct a table (not shown here) and compare directly all the available published data on the survival, implantation and dissemination of Candida species from the gut.

The following generalizations regarding colonization and dissemination of C. albicans and other Candida species from the GI tract are the result of this effort. The foremost generalization that is evident from a comparison of this data is that the indigenous microflora represents one of the most important (if not the most important) host defense mechanisms suppressing the colonization of Candida species in the GI tract. This is clearly demonstrated from several studies that showed conventional animals were significantly more resistant to GI colonization than were

germfree and antibiotic-treated animals (Andermont et al., 1983; Balish et al., 1984; Ekenna & Sherertz, 1987; Guentzel & Herrera, 1982; Helstrom & Balish, 1979; Huppert et al., 1955; Kennedy & Volz, 1983 & 1985 a&b). Another observation that is consistent with this view is that neonatal animals, which lack a complete indigenous microflora, are also readily colonized by C. albicans (Cole et al., 1988; Domer, 1988; Domer & Hector, 1987; Field et al., 1981; Hector & Domer, 1982 & 1983; Pope et al., 1979). The few apparent contradictory results reported in the literature with regard to the effects of the normal intestinal microflora on GI colonization by Candida, may be due to the animals selected for study or to other methodologic pitfalls that accompany the study of interactions between microogranisms in the GI tract. For instance, early findings of Balish and Phillips indicated that C. albicans was established in "large numbers" in the gut of both germfree and conventional mice (Balish & Phillips, 1966; Phillips & Balish, 1966), may be explained by the finding that many strains of mice are unsuitable for research on intestinal floral interactions (Freter et al., 1979; Freter et al., 1983; Kennedy & Volz, 1985b). Such mice are characterized, as are most commercially available strains of mice, by a lack of strictly oxygen-sensitive anaerobes and therfore lack a true indigenous microflora (Freter et al., 1979; Freter et al., 1983; Phillips & Lee, 1984). Using a different strain of mice,

Balish and co-workers (Helstrom & Balish, 1979; Lee & Balish, 1981 & 1982) later reported that conventional animals were significantly more resistant to GI colonization by C. albicans than were antibiotic-treated and germfree mice.

Such data suggest further that the indigenous microflora is also an important defense mechanism inhibiting the systemic spread of Candida from the lumen of the gut to other organs. Animals treated with certain antibiotics, for instance, showed that large populations of C. albicans colonized their GI tracts, with a corresponding high incidence of dissemination to visceral organs (Kennedy & Volz, 1983 & 1985 a&b). Animals that posses a complex indigenous microflora, in contrast, had low numbers of Candida organisms residing in their GI tracts, with a very low percentage or none of the animals showing signs of Candida dissemination. The finding that not all antibiotics predisposed mice to GI colonization or dissemination, may suggest that only certain components of the microflora are primarily responsible for the suppression of Candida in the gut. This will be discussed in more detail below.

It also appears that an intact immune system may also be involved in the suppression of both colonization and dissemination of Candida from the GI tract. This can be seen from the studies of Ekenna and Sherertz (1987) who showed that pretreatment with cyclophosphamide, or

cyclophosphamide and cortisone acetate in combination, predisposed mice to cecal colonization by C. albicans. It should be noted, however, that it has also been shown that mice possessing a competitive bacterial flora are more effective than mice with an intact immune system in suppressing colonization with this fungus (Helstrom & Balish, 1979).

Nonetheless, it may be that local antifungal immunity may act synergistically with bacterial antagonism in controlling C. albicans populations in the intestine. This view is consistent with studies by Shedlofsky and Freter (1974) who showed synergism between ecologic and immunologic control mechanisms regulating bacterial populations in the intestine. Pretreatment of mice with a combination of antibiotics and immunosuppressive agents was found to be more effective than either antibiotics or immunosuppressive agents alone at predisposing the animals to cecal colonization by C. albicans (DeMaria et al., 1976; Ekenna & Sherertz, 1987; Myerowitz, 1981). The possibility that the immunosuppressive agents used in these studies somehow modified the indigenous microflora is not known.

ARTICLES

INFLUENCE OF GROWTH CONDITIONS ON *CANDIDA ALBICANS* ADHESION, HYDROPHOBICITY AND CELL WALL ULTRASTRUCTURE

Michael J. Kennedy, and Ramon L. Sandin

Published in:

The Journal of Medical and Veterinary Mycology

26:79-92, 1988

Influence of growth conditions on *Candida albicans* adhesion, hydrophobicity and cell wall ultrastructure

Michael J. Kennedy[1,2*], and Ramon L. Sandin[2]

[1]Microbiology and Nutrition Research Unit, The Upjohn Company, Kalamazoo, Michigan, 49001, U.S.A., and [2]Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan, 48824, U.S.A.

Running Title: FACTORS INFLUENCING *C. ALBICANS* ADHESION

*Correspondence address: Microbiology and Nutrition Research Unit, The Upjohn Company, Kalamazoo, Michigan, 49001, U.S.A.

ABSTRACT

The effect of cultivation in 13 media (10 complex, and 3 synthetic), as well as altering growth conditions, on Candida albicans adhesion, cell surface hydrophobicity, and cell wall ultrastructure was studied. Adhesion of C. albicans to buccal epithelial cells (BECs) was significantly modified by all of the factors tested, particularly growth medium. In general, optimal adhesive activity for C. albicans was observed when the cells were grown in defined media (depending on the carbohydrate used) and/or at 25°C. Moreover, significant differences in adhesion to BECs were noted when C. albicans was grown in the same complex medium from different manufacturers and in different batches of medium from the same manufacturer. Electron microscopy revealed significant differences in surface topography and cell wall ultrastructure of C. albicans grown in different media, but none of these differences, including presence or absence of an outer floccular layer, appeared to correlate with the adhesive changes noted, which raises questions regarding the location and nature of the Candida adhesin(s). Likewise, cell surface hydrophobicity could not be correlated with adhesion to BECs, but may have influenced yeast coadhesion. The results indicate that Candida adhesion is highly dependent upon the cultivation conditions of the yeast cells tested, and may explain discrepancies in the literature regarding the biochemical

nature of the surface component(s) responsible for C. albicans adhesion.

INTRODUCTION

The adhesion and association of Candida albicans with various host tissues is considered to be an important event in colonization and pathogenesis (9,10,34). Consequently, numerous models (in vitro and in vivo) have been developed to study the adhesion of C. albicans to mucosal surfaces, certain organs, and prosthetic devices (9). These models have led several authors to suggest that various surface components of this yeast mediate adhesion (9,34). It has been suggested, for instance, that a mannose containing moiety, probably a mannoprotein (6,23), is the Candida adhesin (19,35-38), whereas analogous studies have also suggested a possible role for cell wall chitin as the adhesive component (39). Other studies suggest that there may be more than one adhesin (13,23,35), and at least one report suggests that Candida adhesion may not be mediated by a specific adhesin-receptor interaction, but may be entirely nonspecific (31).

One explanation for these different conclusions is the variations in experimental conditions used by different investigators; these include differences in the Candida strain or phenotype, growth and assay medium used, variation in growth conditions (e.g., temperature), and

methods for isolation and preparation of test cells (9,12). However, while all of these factors may have influenced Candida adhesion data, growth conditions are probably the most important since the adhesiveness of microbial cells is directly dependent on the conditions under which the cells are propagated (9). Indeed, Douglas & McCourtie (7,21,22) have already reported that growth of C. albicans in chemically defined media with different amounts and kinds of carbohydrates directly influenced Candida adhesion. While the importance of culture conditions, particularly growth media, on Candida adhesion has been noted in the literature (7,8,14,16,28), it is somewhat surprising to note that over 15 different culture media have been used for propagation of C. albicans for studies of epithelial cell adhesion (9). Of particular interest is the fact that very few investigators have used the same medium, and that there are major differences between media used by those investigators proposing that different cell surface components (adhesins) are responsible for Candida adhesion (9). Consequently, we set out to examine the effects several media, as well as various growth conditions, had on Candida adhesion to buccal epithelial cells, cell surface hydrophobicity and cell wall ultrastructure. Furthermore, for reasons that will become apparent, we have discussed criteria and have suggested what, it is hoped, will be the initial selection of a defined basal medium for future development of a

standardized epithelial cell adhesion assay for C. albicans.

METHODS

Mucosal cells

Buccal epithelial cells (BECs) were collected from 10 healthy adult volunteers by gently rubbing the inside of their cheeks with sterile cotton swabs and suspending the cells in 0.01M phosphate-buffered saline (PBS) at pH 7.2. The donors had no signs or symptoms of oral thrush and had not taken antibiotics for at least 12 months. Cells from the different donors were pooled, washed three times in PBS, resuspended to a concentration of 2×10^5 cells ml^{-1} of PBS, as determined by hemacytometer count and stored at 4°C until required (maximum 2 weeks). Preliminary studies showed that storage of BECs did not affect adhesion of Candida, but controls were included throughout the 2 week experimental period to ensure that differences in adhesion were not due to the use of either fresh or stored BECs. Examination by light microscopy confirmed that none of the cell samples contained mucosal cells already colonized by yeasts.

Yeasts, culture conditions, and cell preparation

C. albicans (AK785), recovered from the oral cavity of a one week-old female with oral thrush, lyophilized and subsequently maintained on Sabouraud's dextrose agar (SDA;

Difco Laboratories, Detroit, MI) at 22°C to 25°C was used throughout the study; it was sub-cultured fewer than six times from the original isolation to minimize changes in adhesive or virulence properties. For all assays, C. albicans was grown on SDA slants at room temperature for 4 days. A loopful of cells was then transferred to 100 ml of test medium and the culture was incubated aerobically (temperature and other growth conditions as stipulated for each medium in Table 1) up to and past the early stationary phase (ESP). Stationary phase cells were used because they adhere more readily than logarithmic phase cells (14,16,38). One portion of the culture was then processed for electron microscopy as follows: Cells were harvested by centrifugation at 3,000 rev. min^{-1} for 10 min at 4°C, washed three times in PBS, and the pellet was gently resuspended in 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) and held at 4°C for 4 h to fix the cells. Following fixation, the cells were centrifuged, and the pellet was gently resuspended in the same buffer without glutaraldehyde and held at 4°C until they were processed for electron microscopy. Another portion of the culture was collected by centrifugation, washed three times in PBS, and resuspended to 2 x 10^7 cells ml^{-1} so as to give a final yeast:buccal cell ratio of 100:1 for use in the adhesion assay. The remainder of the culture was then harvested by centrifugation and washed twice in PUM buffer (22.2 g $K_2HPO_4.3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g

MgSO4.7H2O) (33) and used for hydrophobicity testing.

Adhesion assay

Adhesion of C. albicans to BECs was assayed as described previously (38). Briefly, 0.2 ml samples of BEC and yeast cells were placed into small test tubes (12 x 75 mm) and incubated on a rotary shaker (180 rev. min^{-1}) for 1h at 37°C. Three tubes were used for each experiment. After incubation, BECs were collected and washed on polycarbonate filters (12 μm pore size) (Nucleopore Corporation, Pleasanton, California). Cells were washed with approximately 100 ml PBS under continual (gentle) agitation. The filters were then stained with Gram crystal violet, and the number of C. albicans adhering to 200 BECs was determined by light microscopy (430X magnification). Double-blind conditions were used. Results were read blind, without knowing which medium was being tested. Candida cells grown in TSB + G at 37°C (Table 1) were used as controls with each group of 4/5 media tested to compare adhesion values obtained with the same medium on different days.

Phase-partition test for hydrophobicity

A modification (13) of the phase-partition method of Rosenberg et al. (33), with hexadecane as the hydrocarbon phase (24), was employed to test for Candida hydrophobicity. The relative hydrophobicity was

determined by measuring the difference in absorbances of test and control cells, and determining the percentage of cells that entered the hydrocarbon phase.

Electron microscopy

For scanning electron microscopy (SEM), yeast cells fixed for 4 h in 3% glutaraldehyde in 0.1 M cacodylate buffer, were washed in three changes of buffer, and postfixed in 1% osmium tetroxide in the same buffer for 1 h. The cells were then washed with three changes of buffer, dehydrated by passage through an ethanol series, and critical point dried with a Ladd critical-point dryer. Specimens were coated under vacuum with gold:palladium (60:40) in a Hummer X sputter coater (Technics Inc., Alexandria, VA) and examined in a JEOL JSM-T300 scanning electron microscope at 15 kV. Variations in cell surface features of C. albicans such as mode of budding, bud scar morphology, and surface topography, were examined with respect to growth conditions and compared to previously published reports (3,17). SEM was also used to confirm light microscopy observations on the predominant morphological cell type and the presence of short germ tube-like structures.

The procedure used for transmission electron microscopy (TEM) was a modification of the "G-O-T-O-U" method described by Persi & Burnham (27). Briefly, Candida cells fixed in 3% glutaraldehyde in 0.1 M

cacodylate buffer were postfixed in OsO_4 and tannic acid and were washed three times in cacodylate buffer between each step in the schedule. After fixation, Candida cells were dehydrated by passage through an ethanol series, embedded in Polybed 812, sectioned with a Sorval MT 5000 ultramicrotome (DuPont Co., Wilmington, DE), and poststained with 2% aqueous uranyl acetate and Reynold's lead citrate (32). Sections were then examined in a JEOL-1200 EX transmission electron microscope and photographs taken (at different magnifications) of several fields of cells from each culture. Average cell wall thickness was then determined from the micrographs by measuring the wall thickness at several different points for a number of cells. The presence of a floccular cell wall layer (21,22), whether this was located uniformly or only amorphously around the cell, and the proportion of cells with such a layer was also determined from the micrographs as was the presence of an electron dense outer layer. Finally, the predominant morphological cellular forms, and whether the cells appeared to be germinating, were confirmed by TEM. All micrographs for SEM and TEM were coded, and were examined without knowledge of the growth conditions.

RESULTS

Adhesion of C. albicans to BECs after growth on different media

To determine the effect growth medium had on Candida adhesion to BECs, Candida adhesion after growth in TSB+G was assigned a relative adhesion index of 1, and the adhesion of C. albicans grown on the other 12 different media (Table 1) compared to it. The ability of C. albicans to attach to BECs was found to be highly dependent on the growth medium used (Table 2). In general, Candida cells grown in undefined liquid media at 37°C were usually less adhesive than those cells grown in defined media at the same temperature. However, for one defined medium (YNB), the production of highly adhesive Candida cells was dependent upon the type of carbohydrate in the medium. For example, in assay 15, when glucose was substituted for the galactose in this medium (assay 16), adhesion was reduced. Moreover, significant differences in C. albicans adhesion to BECs were noted when Candida cells were grown in the same complex medium (TSB) from different manufacturers (compare assays 1 & 12), and in different batches of medium from the same manufacturer (compare assays 11 & 12). The ability of C. albicans to attach to BECs after growth in broth or on agar also was tested. It was found that C. albicans grown on Sabouraud's agar at 37°C (assay 19) was significantly more adhesive than cells grown in Sabouraud's broth (assays 4 &

5). Similar results were obtained when the experiments were performed at 25°C, although the differences were not as marked (compare assays 6 and 20).

A strong correlation was also found between C. albicans adhesiveness and the percentage of BECs to which C. albicans had attached (Table 2). As the number of Candida that attached to epithelial cells increased, so did the percentage of BECs which had attached yeasts. The percentage of BECs with one or more attached C. albicans ranged from 28% to 94% after growth of C. albicans in TSB+G-S (assay 2) and LBC-25 (assay 18), respectively.

The ability of C. albicans to associate with BECs indirectly, by attachment to already adherent yeasts, was also studied. It was found that yeast-to-yeast coadhesion contributed to an increase in the total number of C. albicans that attached to BECs after growth in 4 media, namely, LBC, BHI, SDA, and SDB (when grown at 25°C) (Table 2). Coadhesion was also noted after growth in TSB, however, as for adhesion, significant differences were noted, between medium from different manufacturers (compare assays 1 and 11) and between different batches of medium (compare assays 11 and 12).

Effect of growth temperature on C. albicans adhesion

The adhesion of C. albicans to BECs was significantly modified by the temperature at which the cells were grown. Growth at 25°C gave cells that were significantly more

adhesive to BECs than those grown in the same medium at 37°C (Table 2); compare assays 4 & 6, 17 & 18 and 19 & 20. Likewise, coadhesion of C. albicans cells was also found to be greater after growth at 25°C (e.g., assays 4 & 6). Thus, growth temperature, as well as medium, may influence C. albicans coadhesion.

Effect of growth parameters on C. albicans cell surface hydrophobicity

To test the hypothesis that cell surface hydrophobicity may influence the adhesion of C. albicans to BECs, the relative hydrophobic affinity of C. albicans was determined. As is shown in Table 3, the strain of C. albicans used in this study proved to be relatively hydrophilic after growth in 12 of the 13 media tested (at either 37°C or 25°C), since on average only ≤10% of the cells entered the hydrocarbon phase. The only exception was C. albicans grown in LBC at 25°C (assay 18) which was very hydrophobic with about 80% of the cells entering the hydrocarbon phase. Although these latter cells were also the most adhesive (Table 2), no direct correlation was observed between cell surface hydrophobicity and adhesion of C. albicans to BECs. Indeed, at least one half of the total number of attached yeasts were attached indirectly due to yeast coadhesion, with clumps of yeast cells attached to BECs en masse. Therefore, it seems likely that hydrophobic properties contribute little to adhesion

to BECs, but may instead be an important factor in promoting yeast coadhesion; thereby contributing to the total number of C. albicans attached to BECs by indirect adhesion.

Effect of growth conditions on C. albicans morphology, cell wall ultrastructure and surface topography

The morphology and surface features of C. albicans following growth on various test media were examined to determine if certain characteristics could be correlated with the ability of C. albicans to adhere to BECs. The results are summarized in Table 4. Although a number of different morphological characteristics were associated with different experimental groups, only two factors appeared to correlate with increased yeast adhesiveness; namely, the presence of very small blastospores and yeast cells that appeared to be germinating and had short germ tube-like structures.

To determine whether differences in cell wall ultrastructure or various cell surface features could account for some of the differences in Candida adhesion to BECs, cells of C. albicans were examined by SEM and TEM. Although differences were noted in cell wall thickness, number of distinct cell wall layers, and the presence of an extra electron dense cell wall layer by TEM (Fig. 1a), none of these differences appeared to account for either an increase (or decrease) in adhesion, coadhesion, or cell

surface hydrophobicity. Furthermore, the presence or absence of a floccular material on the cell wall, which was previously shown to cause an increase in C. albicans adhesion to BECs (7,22), did not appear to correlate with Candida adhesiveness. Such material was only found in large amounts on cells grown in YNB+gal (Fig. 1b), whereas smaller amounts were present on the cell surface of cells grown in LBC (at 37°C), YNB+G, and TSB+G (Fig. 1c). Growth of C. albicans in the latter two media yielded cells that were relatively nonadhesive to BECs. Furthermore, this material occurred only amorphously on the cell surface and no other cells of C. albicans that were highly adhesive (e.g., assays 10, 12, 13, and 18) possessed such material. Indeed, some highly adhesive cells (e.g., assay 12) of C. albicans appeared to have a relatively thin cell wall with no "extra" material or obvious adhesive appendages (Fig. 1d). To ensure that differences were not due to extra cell wall layers being removed during the preparation of the cells for electron microscopy, two additional procedures (using different fixation methods or stains) were used to examine the cells (data not shown). The results were essentially the same as the results given in Table 4. Thus, the "floccular" material noted for cells grown in YNB+gal may contain only one type of adhesin, that is best expressed after growth in this medium. Examination of TEMs from cells with germ tubes or short germ tube-like structures revealed that

there were major differences in cell wall ultrastructure at the site of germination (Fig. 2). Finally, surface "smoothness" or "roughness," location of bud formation, and shape of the bud scar plug, as determined by SEM, did not appear to correlate with the level of adhesion, coadhesion, or cell surface hydrophobicity observed for C. albicans under the conditions tested (Table 4).

DISCUSSION

The findings presented in this paper clearly demonstrate the importance of growth conditions on the adhesiveness of C. albicans in vitro. Growth of Candida at different temperatures, for instance, revealed that cells grown at 25°C were significantly more adhesive to BECs than cells grown at 37°C and that there was a higher percentage of BECs with attached Candida. This was true for three of four media tested and is similar to the results of Lee & King (19) who showed that C. albicans was significantly more adhesive to vaginal epithelial cells when Candida cells were grown in PPG broth at 25°C compared to cells grown in the same medium at 37°C. Likewise, Segal et al. (39) also found that the percentage of vaginal epithelial cells with attached Candida was higher when C. albicans was grown in YE broth at 28°C compared to cells grown in YE broth at 37°C. Other important variables that were observed to greatly influence Candida adhesion in the present study included

differences in the type, batch, and manufacturer of the medium, whether growth was in liquid medium or on solid medium, and whether the medium was undefined or synthetic (the latter was also influenced by the type of carbohydrate used).

One explanation for the effect of temperature on adhesion noted in this study may be the observation that at the lower temperature the cells with short germ tube-like structures, whereas at the higher temperature they did not (e.g., assays 4 and 6) or did so in lower numbers (e.g., assays 19 and 20). Indeed, this was true whenever C. albicans was found to be highly adhesive, regardless of the growth temperature or medium used. The single exception to this pattern occurred when C. albicans was grown in LBC, in which highly adhesive yeast cells were produced that did not have germ tubes. The finding that most of the highly adhesive cells had germ tubes, therefore, is consistent with previous studies showing that germinated cells of C. albicans were significantly more adhesive than their non-germinated counterparts (15,38,40). It is interesting to further note that for all media that yielded Candida cells that were highly adhesive to BECs, there was no other common morphological, ultrastructural, or physicochemical characteristic (of those parameters tested). Conceivably, then, the induction of germination, singly and collectively with other adhesive factors (9), may have facilitated the

ability of C. albicans to attach to BECs.

It also was found that the increased ability of C. albicans to attach to BECs was accompanied by yeasts attaching to already adherent yeast cells. This coadhesion occurred following growth in several media which yielded cells that were highly adhesive to BECs. Interestingly, yeast cells grown in LBC at 25°C were significantly more adhesive than cells grown in any other medium (at any temperature), manifested the largest amount of coadhesion, and demonstrated significant cell surface hydrophobicity. Furthermore, although no direct correlation was observed between cell surface hydrophobicity and the adhesion of C. albicans to BECs, the property appeared to contribute to the total number of bound yeast by promoting yeast coadhesion. Coadhesion of Candida to yeasts and bacteria has been observed in vivo (13,29), and should, therefore, be examined more closely to determine its role in colonization and pathogenesis of mucosal surfaces.

The differences in adhesion of C. albicans to BECs noted in this study raise questions regarding the location and biochemical nature of the Candida adhesin(s). For instance, at least two morphological classes of Candida adhesins have been noted in the literature, floccular and fibrillar (reviewed in [9]), both of which have been observed to mediate the adhesion of C. albicans in vivo (2,4,25). Alternatively, it has also been shown that

adhesion of C. albicans to epithelial cells was mediated by cells that did not contain such outer surface layers (4). Although it is not realistic to correlate directly the growth conditions of C. albicans in vitro with those encountered in vivo, the present findings suggest that the presence of either floccular or fibrillar structures on the cell surface of C. albicans does not, by itself, indicate that they play a role in adhesion to BECs. Cells grown in TSB+G (assay 2), for instance, were found to contain an outer floccular cell wall layer and were relatively nonadhesive to BECs. It should also be noted that the chemical nature of the "floccular" layer noted between cells grown in various media may be very different. Therefore, some, but not all, such structures may contain Candida adhesins.

In addition, it is interesting to note that differences in cell wall ultrastructure (and presumably cell wall composition [9]) of C. albicans correlated with previous suggestions as to which cell wall component(s) may serve as the Candida adhesin(s). As mentioned above, Segal and co-workers (39) have suggested that cell wall chitin, which is located at the innermost portion of the cell wall (30), may serve as the adhesive component. Cells of C. albicans grown in the medium (YE broth) used by this group yielded cells that possessed the thinnest cell wall with the fewest apparent "layers." Growth of Candida in media (e.g. assay 16) that have been used in

studies suggesting that a mannoprotein on the surface of C. albicans is the Candida adhesin (6,23), in contrast, yielded cells with thicker cell walls that possessed an outer floccular layer. It may be argued then, that at least three different adhesins exist on the surface of C. albicans that mediate its adhesion to BECs: (i) a cell wall adhesin (demonstrated best when cells are grown in LBC), (ii) an adhesin in the floccular layer (YNB+galactose grown cells), and (iii) an adhesin expressed below the surface that becomes evident when the outer electron layer is thinned (YE grown cells). Consistent with the view that there is an adhesive component located below the outer layer(s) is the finding that adhesion of C. albicans to BECs was inhibited by Concanavalin A (Con A), whereas after mannan extraction of Candida cells, Con A did not inhibit the residual, albeit lower, adhesion (35). Nevertheless, the variations in C. albicans adhesion with changes in medium composition also highlight the potential of this organism for cell surface modification in vivo, and offers one explanation why C. albicans has a greater ability to attach to tissues than other Candida species (5,16).

In view of the findings presented in this paper, and the discrepancies regarding which surface component(s) serves as the Candida adhesin(s), the need for a reproducible and defined assay to study C. albicans adhesion is very apparent. The most important variable to

be considered is that of the selection of growth medium. It is clear from this study that uniformity of adhesion results between batches of complex commercial media is unlikely. This is not surprising since peptone based media are highly complex and undefined, and consequently there are major differences in the chemical composition of peptones and "Sabouraud's medium" from laboratory to laboratory, from manufacturer to manufacturer, and from batch to batch from the same manufacturer (26). Therefore, it is proposed here that the medium of Lee et al. (18) or yeast nitrogen base (Difco Laboratories) supplemented with 500 mM galactose (21-23) be used because both media are chemically defined and produce cells of C. albicans that are highly adhesive. Several other factors which are important to the development of a standardized adhesion assay have been described in detail elsewhere (9,34). Nevertheless, further steps toward the standardization of Candida adhesion assays, including determining the phenotypic state of the organism at the time of testing (12), may be necessary if the exact nature of the adhesion to BECs or other surfaces is to be understood.

Acknowledgments

R.L. Sandin's work was supported by a post-doctoral Research Fellowship from Michigan State University.

REFERENCES

1. Anderson, M.L. & Odds, F.C. 1985. Adherence of Candida albicans to vaginal epithelia: Significance of morphological form and effect of ketoconazole. Mykosen 28:531-540.

2. Barnes, J.L., Osgood, W., Lee, J.C., King, R.D. & Stein, J.H. 1983. Host-parasite interactions in the pathogenesis of experimental renal candidiasis. Laboratory Investigations 49:460-467.

3. Brawner, D.L. & Cutler, J.E. 1985. Changes in surface topography of Candida albicans during morphogenesis. Sabouraudia: Journal of Medical and Veterinary Mycology 23:389-393.

4. Calderone, R.A., Lehrer, N. & Segal, E. 1984. Adherence of Candida albicans to buccal and vaginal epithelial cells: Ultrastructural observations. Canadian Journal of Microbiology 30:1001-1007.

5. Critchley, I.A. & Douglas, L.J. 1985. Differential adhesion of pathogenic Candida species to epithelial and inert surfaces. FEMS Microbiology Letters 28:199-203.

6. Critchley, I.A. & Douglas, L.J. 1987. Isolation and

partial characterization of an adhesin from Candida albicans. Journal of General Microbiology 133:629-636.

7. Douglas, L.J., Houston, J.G. & McCourtie, J. 1981. Adherence of Candida albicans to human buccal epithelial cells after growth on different carbon sources. FEMS Microbiology Letters 12:241-243.

8. Kearns, M.J., Davies, P. & Smith, H. 1983. Variability of the adherence of Candida albicans strains to human buccal epithelial cells: Inconsistency of differences between strains related to virulence. Sabouraudia 21:93-98.

9. Kennedy, M.J. 1987. Adhesion and association mechanisms of Candida albicans. In M.R. McGinnis (ed.), Current Topics in Medical Mycology, vol. 2. Springer-Verlag, New York, pp 73-169.

10. Kennedy, M.J. 1987. Role of motility, chemotaxis and adhesion in microbial ecology. Annals of the New York Academy of Science 506:260-273.

11. Kennedy M.J. & Volz, P.A. 1985. Ecology of Candida albicans gut colonization: Inhibition of Candida adhesion, colonization, and dissemination from the gastrointestinal tract by bacterial antagonism. Infection

and Immunity 49:654-663.

12. Kennedy, M.J., Rogers, A.L., Hanselman, L.R., Soll, D.R. & Yancey, R.J. 1988. Variation in adhesion and cell surface hydrophobicity in Candida albicans white and opaque phenotypes. Mycopathologia (in press).

13. Kennedy, M.J., Volz, P.A., Edwards, C.A. & Yancey, R.J. 1987. Mechanisms of association of Candida albicans with intestinal mucosa. Journal of Medical Microbiology 24:333-341.

14. Kimura, L.H. & Pearsall, N.N. 1978. Adherence of Candida albicans to human buccal epithelial cells. Infection and Immunity 21:64-68.

15. Kimura, L.H. & Pearsall, N.N. 1980. Relationship between germination of Candida albicans and increased adherence to human buccal epithelial cells. Infection and Immunity 28:464-468.

16. King, R.D., Lee, J.C. & Morris, A.L. 1980. Adherence of Candida albicans and other Candida species to mucosal epithelial cells. Infection and Immunity 27:667-674.

17. Kulkarni, R.K., Hollingsworth, P.J. & Volz, P.A.

1980. Variation in cell surface features of Candida albicans with respect to carbon sources. Sabouraudia 18:255-260.

18. Lee, K.L., Buckley, H.R. & Campbell, C.C. 1975. An amino acid liquid synthetic medium for development of mycelial and yeast forms of Candida albicans. Sabouraudia 13:148-153.

19. Lee, J.C. & King, R.D. 1983. Characterization of Candida albicans adherence to human vaginal epithelial cells in vitro. Infection and Immunity 41:1024-1030.

20. Liljemark, W.F. & Gibbons, R.J. 1973. Suppression of Candida albicans by human oral Streptococci in gnotobiotic mice. Infection and Immunity 8:846-849.

21. McCourtie, J. & Douglas, L.J. 1981. Relationship between cell surface composition of Candida albicans and adherence to acrylic after growth on different carbon sources. Infection and Immunity 32:1234-1241.

22. McCourtie, J. & Douglas, L.J. 1984. Relationship between cell surface composition, adherence and virulence of Candida albicans. Infection and Immunity 45:6-12.

23. McCourtie, J. & Douglas, L.J. 1985. Extracellular

polymer of Candida albicans: Isolation, analysis and role in adhesion. Journal of General Microbiology 131:495-503.

24. Minagi, S., Miyaka, Y., Fugioka, Y., Tsuru, H. & Suginaka, H. 1986. Cell-surface hydrophobicity of Candida species as determined by the contact-angle and hydrocarbon-adherence methods. Journal of General Microbiology 132:1111-1115.

25. Montes, L.F. & Wilborn, W.H. 1968. Ultrastructure features of host-parasite relationship in oral candidiasis. Journal of Bacteriology 96:1349-1356.

26. Odds, F.C., Hall, C.A. & Abbott, A.B. 1978. Peptones and mycological reproducibility. Sabouraudia 16:237-246.

27. Persi, M. & Burnham, J. 1981. Use of tannic acid as a fixative-mordant to improve the ultrastructural appearance of Candida albicans blastospores. Sabouraudia 19:1-8.

28. Persi, M.A., Burnham, J.C. & Duhring, J.L. 1985. Effects of carbon dioxide and pH on adhesion of Candida albicans to vaginal epithelial cells. Infection and Immunity 50:82-90.

29. Pope, L.M. & Cole, G.T. 1982. Comparative studies of gastrointestinal colonization and systemic spread by Candida albicans and nonlethal yeast in the infant mouse. Scanning Electron Microscopy 4:1667-1676.

30. Poulain, D., Hopwood, V. & Vernes, A. 1985. Antigen variability of Candida albicans. Critical Reviews of Microbiology 12:223-271.

31. Reinhart, H., Muller, G. & Sobel, J.D. 1985. Specificity and mechanism of in vitro adherence of Candida albicans. Annals of Clinical Laboratory Science 15:406-413.

32. Reynolds, E.S. 1963. The use of lead citrate at high pH as electron-opaque stain in electron microscopy. Journal of Cell Biology 17:298-342.

33. Rosenberg, M., Gutnick, D. & Rosenberg, E. 1980. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS Microbiology Letters 9:29-33.

34. Rotrosen, D., Calderone, R.A. & Edwards, J.E. 1986. Adherence of Candida species to host tissues and plastic surfaces. Reviews of Infectious Diseases 8:73-85.

35. Sandin, R.L. 1987. Studies on cell adhesion and concanavalin A induced agglutination of Candida albicans after mannan extraction. Journal of Medical Microbiology 24:145-150.

36. Sandin, R.L. 1987. The attachment to human buccal epithelial cells by Candida albicans: An in vitro kinetic study using concanavalin A. Mycopathologia 98:179-184.

37. Sandin, R.L. & Rogers, A.L. 1982. Inhibition of adherence of Candida albicans to human epithelial cells. Mycopathologia 77:23-26.

38. Sandin, R.L., Rogers, A.L., Patterson, R.J. & Beneke, E.S. 1982. Evidence for mannose-mediated adherence of Candida albicans to human buccal cells in vitro. Infection and Immunity 35:79-85.

39. Segal, E., Lehrer, N. & Ofek, I. 1982. Adherence of Candida albicans to human vaginal epithelial cells: Inhibition by amino sugars. Experimental Cell Biology 50:13-17.

40. Sobel, J.D., Myers, P.G., Kaye, D. & Levison, M.E. 1981. Adherence of Candida albicans to human vaginal and buccal epithelial cells. Journal of Infectious Diseases 143:76-82.

41. Tronchin, G., Poulain, D. & Vernes, A. 1984. Cytochemical and ultrastructural studies of Candida albicans. III. Evidence for modifications of the cell wall coat during adherence to human buccal epithelial cells. Archives of Microbiology 139:221-224.

TABLE 1. Growth conditions used for studying *C. albicans* adhesion, cell surface hydrophobicity, cell wall ultrastructure and various surface features

Experimental assay no.	Medium	Medium abbreviation	Batch no.	Source	Incubation temp. (°C)[a]	Previous references
1	Tryptic soy broth + 4% glucose	TSB+G	732059	Difco	37	(37, 38)
2	Tryptic soy broth + 4% glucose	TSB+G-S	732059	Difco	37	
3	Tryptic soy broth + 4% glucose	TSB+G25	732059	Difco	25	
4	Sabouraud dextrose broth	SDB-1	729945	Difco	37	(12–15, 40)
5	Sabouraud dextrose broth	SDB-2	733616	Difco	37	
6	Sabouraud dextrose broth	SDB-1-25	729945	Difco	25	
7	Sabouraud maltose broth	SMB	710589	Difco	37	
8	Fluid Sabouraud medium	FSM	732259	Difco	37	
9	Mycological broth	MB	703508	Difco	37	
10	Brain heart infusion	BHI	729856	Difco	37	
11	Trypticase soy broth	TSB-1	A8DOWJ	BBL	37	
12	Trypticase soy broth	TSB-2	18DPOE	BBL	37	
13	Phytone peptone + glucose	PPG	E2DJEF	BBL	37	(4, 19)
14	Yeast extract	YE	725192	Difco	37	(39)
15	Yeast nitrogen base + 50 mM glucose	YNB+G	732254	Difco	37	(5, 7, 21–23)
16	Yeast nitrogen base + 500 mM galactose	YNB+gal	732254	Difco	37	(5, 7, 21–23)
17	Lee, Buckley, Campbell + 50 mM glucose	LBC-37			37	
18	Lee, Buckley, Campbell + 50 mM glucose	LBC-25			25	
19	Sabouraud dextrose agar	SDA-37	723254	Difco	37	(1, 41)
20	Sabouraud dextrose agar	SDA-25	723254	Difco	25	

[a]All with shaking except assay no. 2, and agar media (assays 19 & 20).

TABLE 2. Adhesive properties of *C. albicans* grown on different media and under different environmental conditions.

Assay no.	Growth medium[a]	Mean no. *C. albicans* 200 BEC ± SD	Relative adhesion[b]	% BECs with attached *Candida*	Coadhesion[c]
1	TSB+G	142 ± 32	1·0	36	−
2	TSB+G-S	173 ± 25	1·2	28	−
3	TSB+G25	102 ± 38	0·7	32	−
4	SDB-1	152 ± 27	1·1	44	−
5	SDB-2	175 ± 22	1·2	34	−
6	SDB-1-25	622 ± 46	4·3	70	+
7	SMB	104 ± 36	0·7	36	−
8	FSM	147 ± 36	1·0	32	−
9	MB	156 ± 32	1·1	36	−
10	BHI	610 ± 14	4·3	82	+
11	TSB-1	260 ± 16	1·8	56	+
12	TSB-2	671 ± 26	4·7	64	−
13	PPG	443 ± 53	3·1	70	+
14	YE	551 ± 26	3·9	64	−
15	YNB+G	156 ± 29	1·1	36	−
16	YNB+gal	759 ± 87	5·3	76	±
17	LBC-37	579 ± 63	4·1	72	+
18	LBC-25	1233 ± 124	8·7	94	+
19	SDA-37	537 ± 60	3·8	70	+
20	SDA-25	752 ± 135	5·3	76	+

[a]See Table 1 for a complete description of growth medium and other experimental parameters.
[b]Adhesion relative to that of cells grown in TSB+G (assay 1).
[c]Indirect attachment to BEC mediated by yeast-to-yeast coadhesion.

TABLE 3. Hydrophobic properties of *C. albicans* grown on different media and under different environmental conditions

Assay no.	Growth medium[a]	Percentage of cells entering the alkane phase ± SD	Relative hydrophobicity[b]
1	TSB+G	11·1 ± 0·1	1·0
2	TSB+G-S	7·1 ± 0·1	0·6
3	TSB+G25	2·2 ± 3·0	0·2
4	SDB-1	7·2 ± 2·2	0·6
5	SDB-2	0	0
6	SDB-1-25	4·2 ± 0·1	0·4
7	SMB	0	0
8	FSM	8·8 ± 2·3	0·8
9	MB	0	0
10	BHI	14·2 ± 10·1	1·3
11	TSB-1	12·0 ± 0·1	1·1
12	TSB-2	3·6 ± 5·0	0·4
13	PPG	11·1 ± 15·7	1·0
14	YE	13·7 ± 6·4	1·3
15	YNB+G	0	0
16	YNB+gal	4·5 ± 0·1	0·4
17	LBC-37	11·6 ± 5·4	1·1
18	LBC-25	79·2 ± 6·5	7·2
19	SDA-37	12·0 ± 0·1	1·1
20	SDA-25	8·3 ± 0·1	0·7

[a]See Table 1.
[b]Hydrophobicity relative to that of cells grown in TSB+G (assay 1).

TABLE 4. Morphology and surface characteristics of *C. albicans* grown on different media and under different environmental conditions

Assay no.	Growth medium	Morphology[a]	Cell wall ultrastructure			Surface topography[c]		
			Floccular layer	Electron dense layer	Average cell wall thickness (nm)	Surface detail	Budding	Bud scar plug
1	TSB+G	Sy, By	−	−	150	S	MP	Convex
2	TSB+G-S	Sy, By	+	−	200	S	MP	Convex
3	TSB+G25	By, Sy	−	+	240	S	MP	Flat
4	SDB-1	Sy, By	−	−	100	R	MP	Convex
5	SDB-2	Sy, By	ND[b]	ND	ND	ND	ND	ND
6	SDB-1-25	Sy, Gt	ND	ND	ND	ND	ND	ND
7	SMB	Sy, By	−	−	120	S	MP	Convex
8	FSM	Sy, By	−	−	120	S	MP+1	Convex
9	MB	Sy, By	−	+	150	S	MP	Flat
10	BHI	Sy, Gt	−	+	120	S	MP	Flat
11	TSB-1	Sy, By	−	+	150	R	MP	Convex
12	TSB-2	Sy, By, Gt	−	−	100	S	MP	Convex
13	PPG	Pm, Sy, Gt	−	−	180	S	MP	Flat
14	YE	Sy, Gt	−	−	100	S	MP+1	Convex
15	YNB+G	By, Sy	+	−	300	S	MP+1	Flat
16	YNB+gal	Sy, Gt	++	−	275	R	MP	Convex
17	LBC-37	Sy	+	−	140	S	MP	Flat
18	LBC-25	S	−	+	180	S	MP	Flat
19	SDA-37	VSy, Gt	−	−	120	S	MP	Convex
20	SDA-25	SB, VSy, Gt	−	−	100	S	MP	Convex +Flat

[a]Sy = single yeast cells; By = budding yeast cells; Gt = yeast cells with germ tubes or short germ tube-like structures; Pm = pseudomycelium; VSy = single yeast cells of various sizes and shapes; S = 'singlets'; SB = small ('sticky') blastospores. The predominate form is given first.
[b]Not determined.
[c]S = smooth; R = rough, MP = multipolar, 1 = lateral.

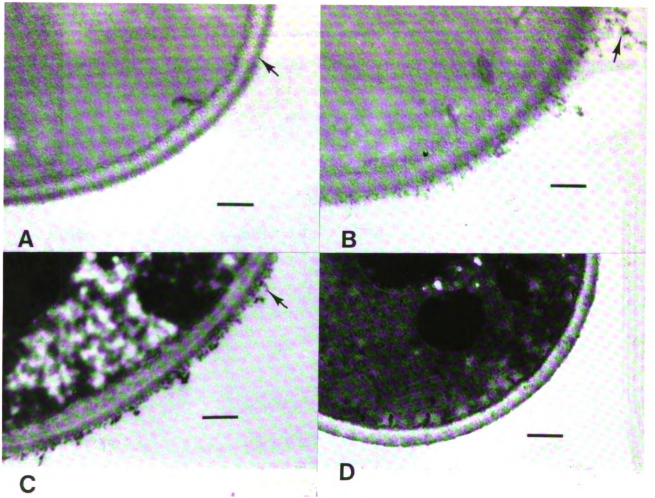


FIG. 1. Electron micrographs of thin sections of *C. albicans* AK785 stained by a modification of the 'G-O-T-O-U' method [37]. Cells harvested from (a) TSB+G25 showing an outer electron dense layer (arrow) (b) YNB+gal showing an outer floccular layer (arrow) (c) TSB+G-S showing an outer floccular layer (arrow) and (d) TSB-2 showing a thin cell wall with no 'extra' cell wall layers or obvious adhesive appendages. Note differences in cell wall ultrastructure using TSB from different manufacturers or different environmental parameters for cell growth. See Table 1 for abbreviations. Bar = 200 nm.

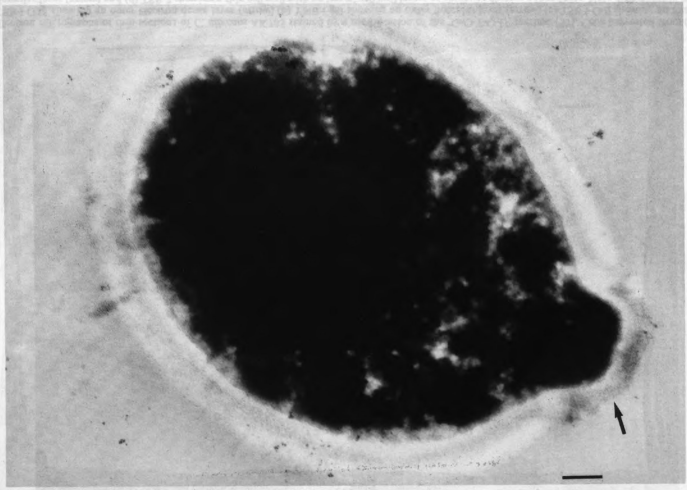


FIG. 2. TEM of thin section of *C. albicans* AK785 showing a tiny germ tube-like structure. Note the alteration of the cell wall ultrastructure at this site (arrow). Bar = 200 nm.

VARIATION IN ADHESION AND CELL SURFACE HYDROPHOBICITY IN *CANDIDA ALBICANS* WHITE AND OPAQUE PHENOTYPES

Michael J. Kennedy, Alvin L. Rogers, Laurey R. Hanselman, David R. Soll, and Robert J. Yancey, Jr.

Published in:

Mycopathologia

102: 149-156, 1988

VARIATION IN ADHESION AND CELL SURFACE HYDROPHOBICITY IN *CANDIDA ALBICANS* WHITE AND OPAQUE PHENOTYPES

Michael J. Kennedy[1,5,*], Alvin L. Rogers[1,2,3], Laurey R. Hanselmen[1,5,+], David R. Soll[4], and Robert J. Yancey, Jr.[5]

Departments of [1]Botany and Plant Pathology, [2]Microbiology and Public Health, and [3]Medical Technology, Michigan State University, East Lansing, Michigan 48824, [4]Department of Biology, University of Iowa, Iowa City, Iowa 52242, and [5]Microbiology and Nutrition Research Unit *(address for correspondence), The Upjohn Company, Kalamazoo, Michigan 49001 USA

Running title: Adhesion of *C. albicans* phenotypes

*Corresponding author

[+]Present address: College of Osteopathic Medicine
Michigan State University, East Lansing, Michigan 48824

ABSTRACT

A previous study had established that a select group of pathogenic isolates of Candida albicans was capable of switching heritably, reversibly and at a high frequency (10^{-2} to 10^{-3}) between two phenotypes ("white" or "opaque") readily distinguishable by the size, shape, and color of colonies formed on agar at 25°C. This paper describes experiments designed to determine the ability of these two phenotypes to attach to buccal epithelial cells (BECs) and plastic, and to compare the cell surface hydrophobicities of white and opaque phenotypes from three clinical isolates. "White cells" were found to be significantly more adhesive to BECs, and a strong correlation was also found between phenotype adhesiveness and the percentage of BECs to which C. albicans had attached. The percentage of BECs with one or more attached C. albicans was approximately 90% for the white phenotype and approximately 50% for the opaque phenotype. "Opaque cells", in contrast, were twice as hydrophobic as white cells, and the percentage of opaque cells bound to BECs by coadhesion was also double that of white cells.

The differences in adhesion to plastic between the two phenotypes were not statistically significant and there was no distinct trend to suggest which phenotype might be more adhesive to plastic. These results indicate that several factors are involved in the adhesion of C.

albicans to plastic, and confirm the hypothesis that cell surface hydrophobicity is of minor importance in direct adhesion to epithelial cells but that it may contribute to indirect attachment to epithelial cells by promoting yeast coadhesion. Moreover, the data presented in this paper also revealed that under identical growth conditions, adhesion of C. albicans was significantly altered depending on the phenotypic state of the organism tested. Therefore, because C. albicans can switch at a high frequency to various phenotypes in vitro, it may be that in future adhesion studies involving Candida the phenotypic state of the organism at the time of testing will have to be determined. Otherwise, the results, even within the same laboratory, may be difficult to interpret.

INTRODUCTION

Recently, it was reported that a select group of pathogenic isolates of Candida albicans was capable of switching heritably, reversibly and at a high frequency (10^{-2} to 10^{-3}) between two general phenotypes ("white" or "opaque") readily distinguishable by the size, shape, and color of colonies formed on agar at 25°C (38). The differences noted in colony characteristics apparently were due to the dramatic difference in cell size, shape, and budding pattern. "White cells" are round to ellipsoidal and exhibit a budding pattern similar to most strains of C. albicans; in contrast, "opaque cells" are elongate, or bean shaped, and exhibit a different budding pattern. While white cells undergo the bud to hypha transition under standard laboratory conditions, opaque cells do not (38). In addition, opaque cells exhibit an unusual pimpled pattern on the cell surface and express an opaque-specific antigen (J.M. Anderson and D.R. Soll, manuscript submitted for publication and unpublished observations). Because the white-opaque transition has now been observed in a number of pathogenic isolates obtained from several body sites (41), it may be that the ability of C. albicans to switch between various phenotypes (37) in vivo may play a role in pathogenicity (38). However, it is unclear what role the opaque phenotype might play since opaque cells are differentially

sensitive to the normal temperature of the human body, at least under laboratory conditions (38). It is possible that the opaque phenotype plays only a transient role in vivo, for instance, if it were more resistant to host defense mechanisms. Alternatively, it may be that the opaque phenotype plays a role in another habitat such as water or in the hospital environment. Such environments would foster a temperature range that would allow for maximal growth of opaque cells. Consequently, we examined cells in the white and opaque phenotypes of several independently isolated strains of C. albicans for adhesion to both buccal epithelial cells and plastic surfaces. Significant differences in adhesion characteristics as well as cell surface properties were observed between these two phenotypes. This report decribes these differences, considers them in relation to recently observed differences in cell surface architecture (J.M. Anderson and D.R. Soll, manuscript submitted for publication and unpublished observations), and discusses the putative role white and opaque phenotypes may play in pathogenesis or alternative habitats.

METHODS

Yeasts, culture conditions, and cell preparation

Three isolates of C. albicans exhibiting a white-opaque transition were recovered from patients with

systemic or vaginal candidiosis. These isolates were used throughout the study and were passed less than six times from the original isolation to minimize changes in adhesive properties. For all assays, C. albicans cells were inoculated onto a modification (37) of the medium of Lee et al. (22), designated here MLBC agar, and grown aerobically at 24°C for four to five days. A colony of cells was transferred to 100 ml of MLBC broth, which was then incubated aerobically with shaking (180 rpm) at 24°C. Cells were grown to stationary phase, and were selected for study because they have been shown to adhere more readily than logarithmic phase cells (18,19,35). One portion of the culture was then processed for adhesion to buccal epithelial cells (BECs). Yeast cells were collected by centrifugation at 3,000 rpm for 20 min at 4°C, washed three times in buffered KCl, and resuspended to 2 x 10^7 cells/ml to give a final yeast:buccal cell ratio of 100:1 for use in the epithelial cell adhesion assay. Another portion of the culture was collected by centrifugation, washed three times in PUM buffer, which consisted of 22.2 g $K_2HPO_4 \cdot 3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$ and distilled water to 1000 ml (29), and resuspended to 5 x 10^6 cells/ml for use in an assay to measure adhesion to plastic. The remainder of the culture was then harvested by centrifugation, washed three times in PUM buffer, and used for hydrophobicity testing at a concentration of 1 x 10^8 cells/ml (17). All

cultures were examined by light microscopy prior to testing to confirm the phenotypic state of the organism.

Mucosal cells

Buccal epithelial cells were collected by gently rubbing the inside of the cheeks of ten healthy adult volunteers with sterile cotton swabs and suspending the cells in 0.05 M KCl containing 1 mM phosphate, 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ ("buffered KCl"; 4) at pH 7.2. This assay medium was chosen because it mimics the ionic composition of saliva (10). The donors were not suffering from signs or symptoms of oral thrush and had not taken antibiotics for at least 12 months prior to the present study. Cells were washed three times in buffered KCl and resuspended to concentrations of 2×10^5 cells/ml of assay medium, as determined by hemacytometer count. This single, large batch of cells was used throughout the study to assure that differences observed in adhesion could be attributed to variables of yeast phenotypic parameters and not to changes or differences in the BECs (16). When examined by light microscopy prior to experimentation, none of the cell samples collected from the different donors contained mucosal cells already colonized by yeasts.

Adhesion assays

The adhesion of C. albicans white and opaque

phenotypes to BECs was studied using a previously described assay (16). Briefly, 0.2 ml samples of BECs and yeast cells were placed into small test tubes (12 x 75 mm) and incubated on a rotary shaker (180 rpm) for 1h at 37°C. Three tubes were used for each experiment. After the incubation period BECs were collected and washed on polycarbonate filters (12 μm pore size) (Nucleopore Corporation, Pleasanton, California). Cells were washed with approximately 100 ml of PBS under continual (gentle) agitation. The filters were then stained with Gram crystal violet and the number of C. albicans adhering to 200 BECs was determined by light microscopy at 430X. Double-blind conditions were used.

The adhesion of C. albicans cells to plastic was studied using polystyrene microtiter trays containing 24, 16 mm-diameter wells (Costar, Cambridge, MA) as follows. A 0.5-ml sample of a suspension of white or opaque Candida cells was placed in each well, the tray incubated for 1.5 h at 37°C without shaking, and the wells then washed three times with 1.0 ml of assay medium. The trays were inverted and allowed to dry overnight at room temperature. Adherent Candida cells were counted by light microscopy at 100X. Five to ten 1 mm^2 fields were counted per well, and the assay was performed in quadruplicate. Only the center area of each well was included in the counting procedure because several nonadherent Candida cells were deposited at the outer edge of the wells during the drying process.

This procedure provided an efficient and reproducible method of quantitating the adhesion of C. albicans to plastic surfaces (M.J. Kennedy, A.L. Rogers, R.V. Thomas, P.A. Volz, and R.J. Yancey, Jr., manuscript submitted for publication).

Phase-partition test for cell surface hydrophobicity

A modification (17) of the phase-partition method of Rosenberg et al. (29), with hexadecane as the hydrocarbon phase, was employed to test for Candida cell surface hydrophobicity. Briefly, yeast were grown as described, washed twice in PUM buffer, and resuspended to a final concentration of 1 x 10^8 yeast/ml. To round-bottom test tubes (12 mm-diameter), containing 2.5 ml of washed cells in PUM buffer, 0.5 ml of hexadecane (Sigma Chemical Co., St. Louis, MO) was added. The suspension was preincubated at 37°C for 10 min, then mixed on a vortex mixer for 2 min. After separation of the aqueous and hydrocarbon phases, the aqueous phase was measured at 400 nm, using a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, NY). Control cells were put through the identical procedure without the addition of hexadecane to the system. The relative cell surface hydrophobicity was then determined by measuring the change in absorbance between test and control cells, and determining the percentage of cells that entered the hydrocarbon phase. This method was selected because of its ease, and because

it has previously been shown to give results similar to all other current methods of testing cell surface hydrophobicity (20,26).

RESULTS

Adhesion of C. albicans phenotypes to BECs

To determine the effect the phenotypic state of C. albicans had on adhesion to BECs, three individual isolates of C. albicans (WO-1, BL6, and 6a) were grown under identical growth conditions in either the white or opaque phenotype (38) and tested by the in vitro assay described in Materials and Methods. To assure that differences were due to phenotype induced changes and not to differences in epithelial cells (7,13,19,33,34), a constant and standardized pool of BECs was used throughout the study (16). The ability of C. albicans to attach to BECs was found to be highly dependent on the phenotype used. These results are summarized in Table 1. For all three isolates, the white phenotype was significantly ($p<0.001$) more adhesive to BECs than was the opaque phenotype. A correlation was also found between phenotype adhesiveness and the percentage of BECs to which Candida cells had attached (Table 1). The percentage of BECs with one or more attached Candida cells was approximately 90% for the white phenotype and approximately 50% for the opaque phenotype. In general, as the mean number of

attached Candida cells increased, so did the percentage of BECs with attached Candida cells. Nevertheless, the white phenotype attached to significantly more BECs than did the opaque phenotype.

The ability of Candida cells to associate with BECs indirectly, i.e. by coadhesion to adherent Candida cells, was also studied. It was found that yeast-to-yeast coadhesion contributed to an increase in the total number of Candida cells that attached to BECs for both phenotypes (Table 1). However, although the percentage of the total coadherent yeast population was low for both phenotypes, opaque phenotypes had approximately twice as many cells attaching to BECs in this manner.

Adhesion of C. albicans phenotypes to plastic

The adhesion of white and opaque Candida cells to plastic was studied using a modification of a microtiter tray assay described by Klotz et al. (20). In preliminary studies (M.J. Kennedy, A.L. Rogers, R.V. Thomas, P.A. Volz, and R.J. Yancey, Jr., manuscript submitted for publication) it was found that two types of adhesion occurred in this system. The first was the direct adhesion of Candida cells to plastic, and the second was adhesion to the liquid-plastic interface. Consequently, as plates dry, cells floating freely in a thin layer of fluid are deposited at the outer edge of the wells. Therefore, to accurately determine the relative number of

Candida cells adherent to the plastic surface, only the center area of the wells were counted. Using this procedure, the adhesion of C. albicans phenotypes to plastic was determined. The results are summarized in Table 2. The differences in adhesion to plastic between the two phenotypes were not statistically significant for any of the isolates tested, and there was no distinct trend to suggest which phenotype was more adhesive to plastic. For instance, for the WO-1-isolate, the white phenotype was more adhesive to plastic than the opaque. In contrast, this trend was reversed for isolate BL6. Furthermore, for isolate 6a the cells of both the white and opaque phenotype attached in similar numbers. This suggests that several factors may be involved in adhesion of C. albicans to plastic surfaces.

Effect of the phenotypic state on C. albicans cell surface hydrophobicity

To test the hypothesis that cell surface hydrophobicity may influence the adhesion of C. albicans to BECs or plastic (11,20), the relative hydrophobic affinity of C. albicans phenotypes was determined. As is shown in Table 3, both the white and opaque phenotypes of the three isolates of C. albicans used in this study proved to be relatively hydrophobic after growth in MLBC at 24°C since about 50% of the cells entered the hydrocarbon phase. Furthermore, it was also noted that

the opaque cells were significantly more hydrophobic than the white cells, with at least twice as many cells adhering to the hydrocarbon phase.

DISCUSSION

It was recently demonstrated that most strains of C. albicans switch heritably and reversibly at high frequency between a number of general phenotypes distinguishable by colony morphology (37-39,41). There are at least three switching systems which are strain specific (40). The "white-opaque transition" is perhaps the most interesting system since it appears to represent a phase transition and involves a dramatic effect on cell shape, cell size, gene expression, growth dynamics, budding dynamics, actin localization and cell wall morphology (38,39; J.M. Anderson and D.R. Soll, manuscript submitted for publication and unpublished observations). Strains which possess a white-opaque transition system are capable of switching back and forth indefinitely between two general phenotypes at frequencies of 10^{-2} to 10^{-3} (39).

The results presented in this report demonstrate that switching between the white and opaque phenotype dramatically influences the adhesive and cell surface properties of individual cells. White cells of three independently isolated strains of C. albicans were significantly more adhesive to BECs than their opaque cell

counterparts. In addition, there was a higher percentage of BECs with attached Candida cells of the white phenotype. This was true for all the isolates tested, and was similar to previous reports that showed a strong correlation to exist between C. albicans adhesiveness and the percentage of BECs to which C. albicans had attached (16,36). In contrast, the opaque cells of each C. albicans isolate tested were far more hydrophobic than their white cell counterparts. The present data, therefore, clearly indicates that under identical growth conditions, adhesiveness, and other cell surface properties, can be significantly different depending on the phenotypic state of the organism tested.

One factor which may be involved in the apparently superior adhesion of white cells to BECs is the differences in size and shape of the phenotypes. White cells are relatively round, whereas opaque cells are elongate, or bean shaped. The mean volume of a population of C. albicans WO-1 white cells was shown to be 33 μm^3, whereas the mean volume of opaque cells was 114 μm^3 (38). Because curved bodies require less kinetic energy to overcome repulsive interactions during adhesion (12,14), such as between C. albicans and epithelial cells (14,30), the smaller, rounder white cells may more effectively reduce the yeast-epithelial gap to allow binding to take place (14). Indeed, it was previously shown that small blastospores of C. albicans were significantly more

adhesive to BECs than larger yeast cells taken from the same culture (16). However, because C. albicans is known to bind essentially irreversibly to BECs (14,15,30), and nonspecific binding is not strong enough to account for this type of adhesion (12), it is likely that specific adhesin-receptor binding was also involved. Although both factors are important in adhesion to epithelial cells, the latter is probably more important since even mild fluid shear can prevent adhesion by dislodging Candida cells that are "attached" only by nonspecific mechanisms (14).

While the data presented here do not allow definitive conclusions to be drawn regarding the increased adhesiveness of white cells to BECs compared to opaque cells, there are, nevertheless, at least two possible mechanisms which could account for this difference. (1) There may be major changes in the organization of the cell wall of these phenotypes (8,14) that allows cells of the white phenotype to produce more or different adhesins that increase their adhesiveness. There is some evidence to suggest that C. albicans does produce more than one type of adhesin (17,24,32), but it is not clear if these types are phenotypically regulated (25) or whether a cell can produce two distinct types of adhesin simultaneously. Although there is no evidence indicating that differences in the composition of the wall exist between white and opaque Candida cells, both scanning and transmission electron microscopy have revealed dramatic differences in

wall morphology (J.M. Anderson and D.R. Soll, manuscript submitted for publication and unpublished observations). The function of "pimples" on opaque cells is still unknown, but such dramatic differences in wall morphology may indeed be related to the difference in adhesion delineated by the BEC adhesion experiments and the hydrophobicity measurements. (2) Because ultrastructural and surface characteristics of white and opaque cells are different, other surface features (e.g., charge) might also be different. Such differences may allow white cells to favor adhesion to BECs by further reducing repulsive interactions that occur during adhesion between cells (14,30). Moreover, such changes may also allow surface molecules adjacent to adhesins to bind nonspecifically to BECs and thereby facilitate and strengthen the adhesion (14).

Factors involved in the adhesion of C. albicans to plastic surfaces were previously investigated by Klotz et al. (20), who found that hydrophobic properties predominately governed Candida-plastic interactions. However, kinetic analyses also revealed negative cooperativity due to electrostatic repulsion (20). Thus, if cell surface hydrophobicity was the only factor involved in the adhesion of Candida to plastic, opaque cells should have attached in higher numbers. The present study, therefore, corroborates the view that other factors are involved in the adhesion of C. albicans to plastic.

Factors other than cell surface hydrophobicity that may have influenced the attachment of C. albicans phenotypes to plastic include cell size and surface charge (14,20).

Previous studies have also shown that C. tropicalis was more hydrophobic and attached in greater numbers to these surfaces than did C. albicans (20,26-28,31). In those studies SDB (at 37°C) was used for cultivation of Candida cells, whereas in this study MLBC (at 24°C) was used. We have found that growth of C. albicans under the latter conditions produced cells that were significantly more hydrophobic (16). The observations of the present study, therefore, were also consistent with previous findings that C. albicans is not very hydrophobic after growth in SDB (16,17,20,26-28), and confirms the suggestion that growth in the medium of Lee et al. (22) at 25°C produces cells that would attach to plastic in the greater numbers (16). Furthermore, the differences in adhesion and coadhesion to BECs noted here for white and opaque phenotypes, corroborates the hypothesis that cell surface hydrophobicity plays only a small role in direct adhesion to BECs but that this property may be important in promoting yeast coadhesion. Opaque cells were generally twice as hydrophobic as white cells, and the percentage of opaque cells bound by coadhesion was also double that of white cells. In contrast, white cells were significantly more adhesive to BECs.

Finally, it is worth considering whether the white-

opaque transition, the resulting differences in adhesion, or both, play a role in colonization and pathogenesis by C. albicans. To begin with, it should be noted that strains exhibiting a white-opaque transition have been isolated from systemic infections, vaginal infections and oral lesions (39). The transition between white and opaque occurs frequently and spontaneously, and appears to involve the activation of opaque specific genes (J.M. Anderson and D.R. Soll, manuscript submitted for publication and unpublished observations). The transition also involves very refined changes in nearly every aspect of growth and cell architecture (38,39), suggesting that the transition is a highly evolved form of hertitable, high frequency variability.

It seems likely, therefore, that this transition does play some role in the pathogenic success of this organism. However, because white cells are significantly more adhesive to epithelial cells than opaque cells, and because white cells have selective growth advantage at 37°C (38), it seems likely that of the two phenotypes the white phenotype would predominate in vivo. Nevertheless, it is possible that the opaque phenotype may play a transient role during the initial phases of colonization, or that the opaque phenotype may be more resistant to irradication by antifungal chemotherapy (B. Slutsky, Ph.D. thesis, University of Iowa; D.R. Soll, M. Staebell, S. Eisely, and B. Slutsky, manuscript in preparation). It is

equally plausable that the dramatic alteration of cell surface properties may offer the opaque phenotype an increased resistance to host defense mechanisms similar to that of encapsulated forms of Cryptococcus neoformans (2,21). Alternatively, the opaque phenotype may predominate in some other environment. Indeed, C. albicans has been isolated from IV lines, hospital linens, soil, water, and toothbrushes (3,5,9). Further studies will be necessary, therefore, to characterize the specific role(s) of the white-opaque transition in pathogenesis, and to determine which phenotypes are present in the host during colonization, invasion, and infection of various body sites. Differences in adhesive properties, nevertheless, may be a major factor in the alternative roles of white and opaque switch phenotypes.

REFERENCES

1. Anderson ML, Odds FC: Adherence of Candida albicans to vaginal epithelia: Significance of morpological form and effect of ketoconazole. Mykosen 28: 531-540, 1985.

2. Breen JF, Lee IC, Vogel FR, Friedman H: Cryptococcal capsular polysaccharide-induced modulation of murine immune response. Infect Immun 36:47-51, 1982.

3. Buck JS: Candida albicans. In: AW Hoadley and BJ

Dutka (eds) Bacterial Indicators/Health Hazards Associated with Water. ASTM STP 635. American Society for Testing and Materials, Philadelphia, 1977, pp 139-147.

4. Clark WB, Bammann LL, Gibbons RJ: Comparative estimates of bacterial affinities and adsorption sites on hydroxyapatite surfaces. Infect Immun 19:846-853, 1978.

5. Cook WL, Schlitzer RL: Isolation of Candida albicans from fresh water and sewage. Appl Environ Microbiol 41:840-842, 1981.

6. Critchley IA, Douglas LJ: Differential adhesion of pathogenic Candida species to epithelial and inert surfaces. FEMS Microbiology Lett 28:199-203, 1985.

7. Davidson S, Brish M, Rubinstein E: Adherence of Candida albicans to buccal epithelial cells of neonates. Mycopathologia 85:171-713, 1984.

8. Douglas LJ: Adhesion of pathogenic Candida species to host surfaces. Microbiol Sci 2:243-247, 1985.

9. Feo M: Supervivencia y desinfection de Candida albicans en el cepillo de dientes. Mycopathologia 74:129-134, 1981.

10. Gibbons RJ, Etherden I, Peros W: Aspects of the attachment of oral streptococci to experimental pellicles. In: SE Mergenhagen and B Rosen (eds) Molecular Basis of Oral Microbial Adhesion. American Society for Microbiology, Washington, 1985, pp 77-84.

11. Hazen KC, Plotkin BJ, Klimas DM: Influence of growth conditions on cell surface hydrophobicity of Candida albicans and Candida glabrata. Infect Immun 54:269-271, 1986.

12. Jones GW, Isaacson RE: Proteinaceous bacterial adhesins and their receptors. CRC Crit Rev Microbiol 10:229-260, 1983.

13. Kearns MJ, Davies P, Smith H: Variability of the adherence of Candida albicans strains to human buccal epithelial cells: Inconsistency of differences between strains related to virulence. Sabouraudia 21:93-98, 1983.

14. Kennedy MJ: Adhesion and association mechanisms of Candida albicans. In MR McGinnis (ed) Current Topics in Medical Mycology, vol 2, Springer-Verlag, New York, 1987, pp 73-169.

15. Kennedy MJ: Role of motility, chemotaxis, and adhesion in microbial ecology. Annal New York Acad Sci

506:260-273, 1987.

16. Kennedy MJ, Sandin RL: Influence of growth parameters on Candida albicans adhesion, hydrophobicity, and cell wall ultrastructure. J Med Vet Mycol 26:79-92, 1988.

17. Kennedy MJ, Volz PA, Edwards CA, Yancey RJ: Mechanisms of association of Candida albicans with intestinal mucosa. J Med Microbiol 24: 333-341, 1987.

18. Kimura LH, Pearsall NN: Adherence of Candida albicans to human buccal epithelial cells. Infect Immun 21:64-68, 1978.

19. King RD, Lee JC, Morris AL: Adherence of Candida albicans and other Candida species to mucosal epithelial cells. Infect Immun 27:667-674, 1980.

20. Klotz SA, Drutz DJ, Zajic FE: Factors governing adherence of Candida species to plastic surfaces. Infect Immun 50:97-101, 1985.

21. Kozel TR: Dissociation of a hydrophobic surface from phagocytosis of encapsulated and non-encapsulated Cryptococcus neoformans. Infect Immun 39:1214-1219, 1983.

22. Lee KL, Buckley HR, Campbell CC: An amino acid liquid synthetic medium for development of mycelial and yeast forms of Candida albicans. Sabouraudia 13:148-153, 1975.

23. McCourtie J, Douglas LJ: Relationship between cell surface composition of Candida albicans and adherence to acrylic after growth on different carbon sources. Infect Immun 32:1234-1241, 1981.

24. McCourtie J, Douglas LJ: Extracellular polymer of Candida albicans: Isolation, analysis and role in adhesion. J Gen Microbiol 131:495-503, 1985.

25. McCourtie J, Douglas LJ: Unequal distribution of adhesins within populations of Candida albicans. FEMS Microbiol Lett 27:111-115, 1985.

26. Minagi S, Miyaka Y, Fugioka Y, Tsuru H, Sunginaka H: Cell-surface hydrophobicity of Candida species as determined by the contact-angle and hydrocarbon-adherence methods. J Gen Microbiol 132:1111-1115, 1986.

27. Minagi S, Miyake Y, Inagaki K, Tsuru H, Suginaka H: Hydrophobic interaction in Candida albicans and Candida tropicalis adherence to various denture base resin materials. Infect Immun 47:11-14, 1985.

28. Miyake Y, Fujita Y, Minagi S, Suginaka H: Surface hydrophobicity and adherence of Candida to acrylic surfaces. Microbios 46:7-14, 1986.

29. Rosenberg M, Gutnick D, Rosenberg E: Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS Microbiol Lett 9:29-33, 1980.

30. Rotrosen D, Calderone RA, Edwards JE: Adherence of Candida species to host tissues and plastic surfaces. Rev Infect Dis 8:73-85, 1986.

31. Rotrosen D, Gibson TR, Edwards JE: Adherence of Candida species to intravenous catheters. J Infect Dis 147:594, 1983

32. Sandin RL: Studies on cell adhesion and concanavalin A induced agglutination of Candida albicans after mannan extraction. J Med Microbiol (in press), 1987.

33. Sandin RL, Rogers AL, Beneke ES, Fernandez MI: Influence of mucosal cell origin on the in vitro adherence of Candida albicans: Are mucosal cells from different sources equivalent? Mycopathologia 98:111-119, 1987.

34. Sandin RL, Rogers AL, Fernandez MI, Beneks ES:

Variations in affiity to Candida albicans in vitro among human buccal epithelial cells. J Med Microbiol (in press), 1987.

35. Sandin RL, Rogers AL, Patterson RJ, Beneke ES: Evidence for mannose-mediated adherence of Candida albicans to human buccal cells in vitro. Infect Immun 35:79-85, 1982.

36. Segal E, Lehrer N, Ofek I: Adherence of Candida albicans to human vaginal epithelial cells: Inhibition by amino sugars. Exp Cell Biol 50:13-17, 1982.

37. Slutsky B, Buffo J, Soll DR: High frequency switching of colony morphology in Candida albicans. Science 230:666-669, 1985.

38. Slutsky B, Staebell M, Anderson J. Risen L, Pfaller M, Soll DR: "White-opaque transition": A second high-frequency switching system in Candida albicans. J Bacteriol 169:189-197, 1987.

39. Soll DR: High frequency switching in Candida. In DE Berg and MM Howe (eds) Mobile DNA. American Society for Microbiology, Washington, 1988, in press.

40. Soll DR, Langtimm CJ, McDowell J, Hicks J, Galask R:

High-frequency switching in Candida strains isolated from vaginitis patients. J Clin Microbiol 25:1611-1622, 1987.

41. Soll DR, Slutsky B, Mackenzie S, Langtimm C, Staebell M: Two newly discovered switching systems in Candida albicans and their possible roles in oral candidiasis. In IC Mackenzie and CA Squier (eds) Diseases of the Oral Mucosa. Published for Dows Institute for Dental Research by Laegeforeningens Folarg, Denmark, 1987, in press.

Table 1. Adhesion of Candida albicans white and opaque phenotypes to buccal epithelial cells (BECs).

Strain	Phenotype	Mean no. yeast per BEC ± SD	% of yeast co-attached	% of BECs with yeast attached
WO-1	White	14.9 ± 5.8	6.1	92.0
	Opaque	2.1 ± 3.3	12.2	50.0
BL-6	White	13.5 ± 3.4	12.8	88.7
	Opaque	1.9 ± 4.7	27.5	31.2
6a	White	18.2 ± 5.8	13.8	96.0
	Opaque	3.0 ± 5.3	23.2	44.0

Table 2. Adhesion of Candida albicans white and opaque phenotypes to plastic.

Strain	Phenotype	Mean no. yeast per mm^2 ± SD
WO-1	White	154.2 ± 31.8
	Opaque	94.8 ± 41.3
BL-6	White	76.2 ± 12.4
	Opaque	110.8 ± 26.0
6a	White	62.7 ± 30.1
	Opaque	76.1 ± 33.6

Table 3. Hydrophobic properties of *Candida albicans* white and opaque phenotypes.

Strain	Phenotype	% change in absorbance ± SD
WO-1	White	45.2 ± 1.1
	Opaque	82.7 ± 3.8
BL-6	White	53.2 ± 1.3
	Opaque	80.0 ± 0.5
6a	White	40.3 ± 1.6
	Opaque	96.8 ± 0.5

ENVIRONMENTAL ALTERATION AND EVIDENCE FOR TWO DISTINCT MECHANISMS OF *CANDIDA ALBICANS* ADHESION TO PLASTIC

Michael J. Kennedy, Alvin L. Rogers,

and Robert J. Yancey, Jr.

Submitted for publication to:

Infection and Immunity

October 10, 1988

Environmental alteration and evidence for two distict mechanisms of *Candida albicans* adhesion to plastic

Michael J. Kennedy[1,4*], Alvin L. Rogers[1,2,3], and Robert J. Yancey, Jr.[4]

Departments of [1]Botany and Plant Pathology, [2]Microbiology and Public Health, and [3]Medical Technology, Michigan State University, East Lansing, Michigan 48824, and [4]The Upjohn Company, Kalamazoo, Michigan 49001

Running title:

*Correspondence address:

The Upjohn Company
Microbiology and Nutrition Research
7922-190-MR
Kalamazoo, Michigan 49001

ABSTRACT

Few studies on the adhesion of Candida albicans to nonbiologic surfaces have been conducted in standardized systems. In this study, the adsorption of C. albicans to plastic was examined after growth in two chemically defined media, Lee-Buckley-Campbell (LBC) and yeast nitrogen base (YNB), using a standardized microtiter tray assay. Two types of adhesion were found to occur in this system: (i) direct adsorption to plastic and (ii) adsorption to the plastic-liquid interface. The adhesion of C. albicans to plastic only was subsequently analyzed by binding isotherms, Langmuir isotherms, and Scatchard plots. The number of binding sites (N) and the affinity constants (K) were calculated. The K and N were twofold and fourfold higher, respectively, after growth in LBC compared to YNB. Scatchard curves for both LBC and YNB grown cells had negative slopes, which is supportive evidence for the view that negative cooperativity is involved in the binding process. A comparison of different assay solutions gave similar results, with the solution given to dehydrated patients (D5.45) allowing for the highest K and the largest N. Additional experiments to examine the role of cell surface hydrophobicity (CSH) in adsorption to plastic were conducted using white and opaque phenotypes of C. albicans. These studies showed that there was no significant difference in the adsorption of these phenotypes to plastic, although the opaque

phenotype was significantly more hydrophobic. Adsorption but not CSH of both phenotypes was significantly greater in D5.45. Moreover, relatively hydrophilic mycelial forms of C. albicans were found to attach only when D5.45 was used as the assay medium. In addition, yeast phase cells, but not mycelial forms, were sensitive to reduction in adsorption by non-ionic detergents. These results suggest that there may be at least two distinct mechanisms of adsorption of C. albicans to plastic, and that growth and environmental parameters can have a profound effect on this process.

INTRODUCTION

The "attachment" of Candida albicans to various biomaterials and host tissues has been deemed an important step in the initiation of both superficial and deep seated candidosis (5). Adhesion of C. albicans to various plastic surfaces that comprise catheters, prosthetic cardiac valves, and other protheses, for example, may allow this opportunistic fungal pathogen: (i) a direct entrance into the vascular system through a breach in vascular patency, such as is caused by an indwelling catheter; (ii) to establish a reservoir for chronic inoculation and thereby dissemination of fungal cells; (iii) to damage tissue cells adjacent to colonized biomaterials; and (iv) to become more resistant to host defenses and antifungal therapy (5). The ability of C. albicans to adhere to intravenous catheters, various plastics and acrylic surfaces has been documented (6,10, 13-15).

Although biomaterials are being used with increasing frequency for tissue substitution (3), relatively few studies on the adhesion of C. albicans and other Candida species to plastic surfaces have been reported (5). In one study, the adhesion of Candida species was examined microscopically after "colonized" catheters were removed from patients in an attempt to obtain information on the morphologic nature of the attached fungi (12). In another study, attached Candida species were examined

quantitatively and were shown to adhere to polyvinyl chloride and Teflon catheters in large numbers *in vitro* (17). Factors that might govern the adhesion of *Candida* cells to plastic were also examined by Klotz *et al.* (10) and it was suggested that hydrophobic properties of the yeast and/or the plastic surface predominately governed the adhesion of *C. albicans* to plastic. Other factors were also examined, but two different, nonstandardized assay methods were used. Furthermore, Sabouraud dextrose broth, which is an undefined peptone based medium that produces cells of relatively low cell surface hydrophobicity (CSH) (4,7,15), was used for cell growth (10). Therefore, in the present study the adhesion of *C. albicans* to plastic was examined after growth in two synthetic media; one of which produces cells of high CSH (LBC) and one that produces cells of relatively low CSH (YNB). Using this approach it was found that two distinct mechanism are involved in the adhesion of *C. albicans* to plastic.

MATERIALS AND METHODS

Fungi. *C. albicans* AK785, recovered from the oral cavity of a 1 week-old female with oral thrush, lyophilized and subsequently stored in Sabouraud dextrose broth (Difco) supplemented with glycerol (30% final concentration) at -74°C, was used throughout the study

except where noted. The organism was subcultured fewer than five times from the original isolation to minimize changes in adhesive or virulence properties. In addition, five isolates recovered from the blood of patients with systemic candidosis were also used (BL-6, CA30, CA34M, WO-1, and 6a).

Growth conditions. Unless otherwise noted, yeast phase cells of C. albicans were grown aerobically at 25°C in the medium of Lee et al. (LBC; 11) or yeast nitrogen base (YNB; Difco) (13) supplemented with either 50mM glucose or 500mM galactose. Cells were grown to stationary phase (48 h), and were selected for study because they have been shown to adhere more readily than logarithmic phase cells (9). For growth of white and opaque cells, C. albicans cells were inoculated onto a modification (18,19) of LBC, designated here MLBC agar, and grown aerobically at 24°C for four to five days. A colony of cells was transferred to 100 ml of MLBC broth, which was then incubated aerobically with shaking (180 rpm) at 24°C. Cells of white and opaque phenotypes were grown to stationary phase. In addition, a mycelial form of isolate 6a arose spontaneously and was also tested.

Cell preparation. After cell growth, yeasts were collected by centrifugation, and the pellet was gently washed twice in buffered KCl (1,2,5), phosphate buffer (pH

7.0), or a variation of one of these buffers. The cells were suspended to give a final concentration of 5 X 10^6 Candida per ml. A range of cell concentrations (1 x 10^5 to 1 x 10^8 cells per ml) was also tested to study Candida binding kinetics.

Adhesion assay. The adhesion of C. albicans cells to plastic was studied using polystyrene microtiter trays (Costar, Cambridge, MA) containing 48, 11 mm-diameter wells as outlined in Figure 1. Briefly, a 0.5 ml sample of a suspension of Candida cells was placed in each well, the tray incubated for 1 h at 25°C without shaking, and the wells then washed three times with 1.0 ml of assay medium. After incubation of the assay mixture and washing, the trays were inverted and allowed to dry overnight at room temperature. Adherent C. albicans were counted by light microscopy, without staining, at 100x. Five to ten 1 mm^2 fields were counted per well, and the assay was performed at least six times. Only the center area of each well was included in the counting procedure because several nonadherent C. albicans cells were deposited at the outer edge of the wells during the drying process. Occasionally, clear patches with few or no yeast cells were observed around or near the center of the well, presumably due to static, and were not included in the areas to be counted. This method provided an efficient and reproducible assay for quantitating the adhesion of C.

albicans to plastic (6).

Phase-partition test for cell surface hydrophobicity. A modification of the phase-partition test of Rosenberg et al. (16) was used as described previously (8).

RESULTS

Influence of growth and assay media on adsorption. To determine the effect growth or assay medium had on the adsorption kinetics of C. albicans to plastic, a wide range of yeast cells were examined for their ability to adsorb to plastic after growth in LBC or YNB and the cells were washed in one of several buffers. The number of C. albicans cells which adsorbed to the plastic when suspended in D5.45 varied significantly according to the growth medium used, although the concentration of cells at which saturation of the available binding sites occurred was the same (Figure 2). The concentration of C. albicans which saturated the available binding sites in this assay medium was 5×10^7. The number of bound yeast cells increased in a curviliner fashion, but a dip in an otherwise smooth curve became evident at concentrations beyond the equilibrium point. When these experiments were repeated in another buffer, namely PBS, the concentration at which the available binding sites became available was 1×10^7 cells per ml (data not shown). Nevertheless, the

adsorption isotherms followed the same curviliner pattern, dipping at concentrations above the equilibrium point. This appeared to be due to yeast cells aggregating during the assay. In all assays various degrees of yeast aggregation became evident at concentrations above 5×10^6 cells/ml, but the degree of yeast aggregation was dependent upon the growth and assay medium used (data not shown). Because of this, a cell concentration of 5×10^6 cells/ml was chosen as the standard cell concentration for the remainder of the studies.

The number of binding sites (N) and the affinity constant (K) were calculated for each growth and assay medium (Table 1). The values for these adsorption parameters varied considerably depending upon the growth and assay medium used. Yeast cells grown in YNB were usually less adhesive than those grown in LBC, regardless of medium carbohydrate or assay medium. The K and N were two-fold and four-fold higher, respectively, after growth in LBC compared to YNB. A comparison of different assay solutions gave varying results, with the solution given to dehydrated patients (D5.45) allowing for the highest K and the largest N (Table 1). The product of the K and N for each growth and assay medium was also calculated and the KN for D5.45 was on average two-fold higher than for PBS. Similarly, the KN for C. albicans grown in LBC supplemented with glucose or galactose were three- to ten-fold higher than those cells grown in YNB, respectively

(Table 1).

These data were also prepared as a Scatchard plot to determine if positive or negative cooperativity influenced binding to plastic (Figure 3). It was noted that a dip in the curves for cells grown in LBC and YNB occurred regardless of the carbohydrate used. The bowing of each line in the Scatchard plot indicated that adsorption to plastic under the conditions used in the present study displayed negative cooperativity. However, at cell concentrations between 5 x 10^7 and 1 x 10^8 cells per ml, positive cooperativity was noted (data not shown).

Influence of assay medium on adsorption. The effect of various assay media on adsorption of C. albicans to plastic was determined and the results are shown in Table 2. The optimal assay medium was D5.45, and this was true regardless of the medium in which the cells were grown. Comparison of several assay media suggested that adsorption of C. albicans to plastic is a multifactoral process that can be manipulated by several factors. For instance, when LBC supplemented with 50 mM glucose was used as the growth medium, yeast cells adhered in significantly greated numbers when Mg^{+2} ions were added to D5.45. This trend held true when other assay media were employed in the adsorption assay (data not shown). A comparison of adsorption ability was also made by comparing the number of adherent yeast cells when

variations of a particular assay medium was used in the adsorption assay. D5.45, for instance, was split into its component parts (i.e., 5% glucose and .45% NaCl) and it was observed that adhesion of C. albicans AK785 was significantly lower when D5.45 was used as the assay medium.

The ionic strength of the medium as well as the relative adhesion strength and average cell size are also listed in Table 2. From these data it appeared that as the ionic strength of the assay buffer increased, the adsorption of Candida cells to plastic decreased. However, because several factors can apparently influence the adsorption of C. albicans to plastic, and because of the large differences between the assay media used, the influence ionic strength had on adhesion was examined more closely.

Influence of ionic strength and ions on adsorption. The effect of KCl concentration on adsorption to plastic is shown in Figure 4. The buffer was 2 mM phosphate, pH 7.0. As is shown in the figure, adsorption of C. albicans to plastic decreased as the ionic strength of the assay buffer increased. The effect various concentrations of ions (Ca^{+2} and Mg^{+2}) had on the attachment of C. albicans to plastic is shown in Figure 5. All concentrations of calcium and magnesium ions increased adsorption to plastic.

Influence of detergents on adsorption. Adsorption of C. albicans CA34M to plastic was reduced by 90% and 60%, respectively, when Tween 80 (TW) or Triton X-100 (TX) were included in the assay suspension, whereas the inclusion of either sodium dodecyl sulfate (SDS) or cetyltrimethylammonium chloride (CTMAC) to similar preparations had no effect (Figure 6). A similar pattern was observed for C. albicans CA30 in that the inclusion of TW or TX reduced adsorption to plastic, but SDS or CTMAC did not (Figure 6). In contrast, no reduction in adsorption was noted when mycelial cells of C. albicans CA30 were used in the assay, regardless of the detergent used (Figure 6).

Adsorption and cell surface hydrophobicity of white and opaque phenotypes. The adsorption of white and opaque phenotypes to plastic was determined in two different buffers. The results are summarized in Table 3. The differences in adsorption to plastic between the two phenotypes were statistically significant, but there was no trend to suggest which phenotype was more adhesive to plastic. For instance, for the WO-1 isolate, the white phenotype was more adhesive to plastic than was the opaque. In contrast, this trend was reversed for isolate BL6, whereas for isolate 6a the cells of both the white and opaque phenotypes attached in similar numbers. Furthermore, a mycelial form of isolate 6a attached to

plastic only when D5.45 was used as the assay medium. These results suggest that several factors may be involved in the adsorption of C. albicans to plastic surfaces.

To test the hypothesis that CSH is the primary mechanism of adhesion of Candida cells to plastic, the relative cell surface hydrophobicities of C. albicans white, opaque, and mycelial phenotypes were determined. As is shown in Table 3, both the white and opaque phenotypes of three isolates of C. albicans used in this study proved to be relatively hydrophobic after growth in MLBC at 24°C since on average 40% or more of the cells entered the hydrocarbon phase. The only exception to this was for mycelial cells of isolate 6a, which were relatively hydrophilic. Furthermore, it was also noted that the opaque cells were significantly more hydrophobic than the white cells, with at least twice as many cells adhering to the hydrocarbon phase. In addition, there was no difference in the relative CSHs of the phenotypes when the assay was performed in PUM versus D5.45, with the single exception of white cells of isolate WO-1 which were slightly more hydrophobic in D5.45 (Table 3).

DISCUSSION

Adsorption of microorganisms to nonbiological surfaces has been proposed to occur in two phases (5). The first is the nonspecific, reversible phase, which is

achieved by reaching an equilibrium between the attraction of London-Van der Waals forces and the repulsion of electrostatic forces. During this initial phase, microorganisms are susceptible to environmental alterations which influence electrostatic and other nonspecific forces (5). The second or specific phase of adsorption involves the bridging of cells to the surface through the interaction of macromolecules and leads to firm adhesion (5). In the current report, as well as other studies on microbial adsorption, it is probably more appropriate to describe adsorption as occurring in three phases: (i) adsorption to the liquid-plastic interface, (ii) initial or reversible adsorption to plastic, and (iii) irreversible or "permenant" adhesion.

The adsorption properties of C. albicans to plastic in the present report were found to be very different under several environmental conditions tested as well as due to the phenotypic state of the organism at the time of testing. Collectively, the data presented here suggest that: (i) the reversible phase of attachment greatly influences the final adsorption of C. albicans to plastic, since those environmental parameters which altered electrostatic forces interfered with irreversible attachment, (ii) several factors can modify adsorption of C. albicans to plastic, and (iii) C. albicans can attach to plastic by more than one mechanism.

Repulsive electrostatic forces between C. albicans

and plastic surfaces have been proposed to be minor in comparison with hydrophobic forces (10), suggesting that the adsorption of yeast cells to plastic should either be altered little by increasing the ionic strength or that this change should result in a greater adsorption by "salting-out" hydrophobic surface moieties (20). When the ionic strength of the assay buffer was increased, however, a reduction in the number of adsorbed yeasts was noted. In contrast, the addition of Ca^{+2} or Mg^{+2} ions to the assay buffer increased the adsorption of C. albicans to plastic. This is in contrast to the findings of Klotz et al. (10) who found that increasing the ionic strength of the assay buffer or altering the yeast surface charge to be more positive led to an increase in the number of yeast cells that adsorbed to plastic.

It is not clear, however, whether the differences noted from the present study and that of Klotz et al. (10) are due to differences in the "strains" of C. albicans used or to differnces in experimental conditions. In the present study, the medium of Lee et al. (11) was used for cell growth, whereas in the study reported by Klotz and co-workers yeast cells were grown in SDB. Yeast phase cells of C. albicans grown in these two media have been shown to very greatly in both their CSH and cell wall ultrastructure (7). This alone could account for the differences noted between the results of experiments examining the influence of ionic strength on the

adsorption to plastic, in that cells grown in LBC are very hydrophobic whereas those grown in SDB are relatively hydrophilic (7,15). Indicating that there were fewer hydrophobic moieties to be salted-out in LBC-grown cells, that cells of C. albicans that are very hydrophobic are more influenced by electrostatic interactions during binding, or both. The finding that there were little difference in the CSHs between yeast cells washed and suspended in PUM, PUM + NaCl, D5.45, 0.45% NaCl, and distilled water, but that there were differences in adsorption to plastic in D5.45 is certainly in agreement with this. Thus while these data tend to confirm the importance of hydrophobic properties of the yeast cell surface in adsorption to nonbiological surfaces (5,10), these data also suggest that other factors may be of equal or greater importance in adsorption depending upon the prevailing environmental parameter.

The use of detergents in adsorption assays produced additional data concerning view and the role electrostatic and hydrophobic interactions may play in the adsorption of C. albicans with plastic. For instance, the adsorption of yeasts to plastic was not influenced by the presence of ionic detergents (i.e., CTMAC or SDS), but was reduced significantly by the presence of the non-ionic detergents Tween-80 and Triton X-100. The latter two detergents interfer with hydrophobic binding. Thus suggesting that CSH is the predominant force governing the adsorption of

yeast phase cells of C. albicans to plastic. However, while CSH is certainly a major adhesive force involved in the attachment of yeasts to plastic, it is not the only one. Studies with white and opaque phenotypes of C. albicans are consistent with this view. Thus if increased CSH alone were responsible for adsorption to plastic, opaque cells should have been found to attach in greater numbers. The finding that there was no distinct pattern to suggest which phenotype is more adhesive to plastic, although opaque cells were significantly more hydrophobic, confirms the suggestion that several factors collectively are responsible for adsorption of C. albicans to nonbiological surfaces (6).

One factor which may be involved in the superior adsorption of yeasts in certain buffers is the differences in size and shape of the cells. Cells that are relatively round in shape and smaller in size, for instance, may require less kinetic energy to overcome repulsive interactions during adsorption (5). In addition, it appears that there is more than one mechanism by which C. albicans binds to plastic. A mycelial form of C. albicans 6a, for instance, was found to be relatively hydrophobic in both D5.45 and PUM buffers (which should excentuate CSH), but was found to attach in significantly higher numbers in D5.45. In addition, the adsorption of a mycelial form of strain CA30 was not influenced by the the incorporation of either ionic or non-ionic detergents into

the assay.

Collectively, these results suggest that there may be at least two distinct mechanisms of adsorption of C. albicans to plastic, one of which appears to be hydrophobic in nature and another which may be mediated by an ion-bridge type of mechanism, and that growth and environmental parameters can have a profound effect on these processes. These data also underscore the great variability that can arise from the use of "simple" adhesion assays, and suggest that standardization of such assays as well as determining the phenotypic state of the organism at the time of testing may be required if meaningful results are to be obtained between and within laboratories (5,6).

REFERENCES

1. Clark WB, Bammann LL, Gibbons RJ: Comparative estimates of bacterial affinities and adsorption sites on hydroxyapatite surfaces. Infect Immun 19:846-853, 1978.

2. Gibbons RJ, Etherden I, Peros W: Aspects of the attachment of oral streptococci to experimental pellicles. In: SE Mergenhagen and B Rosen (eds) Molecular Basis of Oral Microbial Adhesion. American Society for Microbiology, Washington, 1985, pp 77-84.

3. Gristina AG: Biomaterial-centered infection: microbial

adhesion versus tissue integration. Science 237:1588-1595, 1987.

4. Hazen KC, Plotkin BJ, Klimas DM: Influence of growth conditions on cell surface hydrophobicity of Candida albicans and Candida glabrata. Infect Immun 54:269-271, 1986.

5. Kennedy MJ: Adhesion and association mechanisms of Candida albicans. In MR McGinnis (ed) Current Topics in Medical Mycology, vol 2, Springer-Verlag, New York, 1987, pp 73-169.

6. Kennedy MJ, Rogers AL, Hanselman LR, Soll DR, Yancey RJ: Variation in adhesion and cell surface hydrophobicity in Candida albicans white and opaque phenotypes. Mycopathologia 102: 149-156, 1988.

7. Kennedy MJ, Sandin RL: Influence of growth parameters on Candida albicans adhesion, hydrophobicity, and cell wall ultrastructure. J Med Vet Mycol 26:79-92, 1988.

8. Kennedy MJ, Volz PA, Edwards CA, Yancey RJ: Mechanisms of association of Candida albicans with intestinal mucosa. J Med Microbiol 24:333-341, 1987.

9. Kimura LH, Pearsall NN: Adherence of Candida albicans

to human buccal epithelial cells. Infect Immun 21:64-68, 1978.

10. Klotz SA, Drutz DJ, Zajic FE: Factors governing adherence of Candida species to plastic surfaces. Infect Immun 50:97-101, 1985.

11. Lee KL, Buckley HR, Campbell CC: An amino acid liquid synthetic medium for development of mycelial and yeast forms of Candida albicans. Sabouraudia 13:148-153, 1975.

12. Locci R, Peters G, Pulverer G: Microbial colonization of prosthetic devices. IV. Scanning electron microscopy of intravenous catheters invaded by yeasts. Zentralbl Bakteriol Hyg (B) 173:419-424, 1981.

13. McCourtie J, Douglas LJ: Relationship between cell surface composition of Candida albicans and adherence to acrylic after growth on different carbon sources. Infect Immun 32:1234-1241, 1981.

14. Minagi S. Miyake Y, Inagaki K, Tsuru H, Suginaka H: Hydrophobic interaction in Candida albicans and Candida tropicalis adherence to various denture base resin materials. Infect Immun 47:11-14, 1985.

15. Miyake Y, Fujita Y, Minagi S, Suginaka H: Surface

hydrophobicity and adherence of Candida to acrylic surfaces. Microbios 46:7-14, 1986.

16. Rosenberg M, Gutnick D. Rosenberg E: Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS Microbiol Lett 9:29-33, 1980.

17. Rotrosen D, Gibson TR, Edwards JE: Adherence of Candida species to intravenous catheters. J Infect Dis 147:594, 1983

18. Slutsky B, Buffo J, Soll DR: High frequency switching of colony morphology in Candida albicans. Science 230:666-669, 1985.

19. Slutsky B, Staebell M, Anderson J. Risen L, Pfaller M, Soll DR: "White-opaque transition": A second high-frequency switching system in Candida albicans. J Bacteriol 169:189-197, 1987.

20. Smyth CJ, Jonsson P, Olsson E, Söderlind O, Rosengren J, Hjerten S, Wadstrom T: Differences in hydrophobic surface characteristics of porcine enteropathogenic Escherichia coli with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect Immun 22:462-472, 1978.

Table 1.

Comparison of binding affinities and adsorption sites for *Candida albicans* after growth in different media or with different assay media

Growth Medium	Assay Medium	Affinity Constant (K) ($X10^{-5}$)	No. of binding sites (N) per well ($X10^{3}$)	KN ($X10^{-2}$)
LBC + glucose	PBS	2.0	5.5	11.0
	D5.45	3.0	5.4	16.2
LBC + galactose	PBS	2.5	6.2	15.5
	D5.45	4.1	9.0	36.9
YNB + glucose	PBS	1.2	2.5	3.0
	D5.45	1.8	3.3	5.9
YNB + galactose	PBS	2.4	2.0	4.8
	D5.45	1.0	3.6	3.6

Table 2.

Effect of assay medium on the adsorption of *Candida albicans* to plastic

Assay Medium	Ionic Strength	Mean No. Candida / mm^2 ± SD after growth in:	
		LBC + 50 mM glucose	YNB + 50 mM glucose
Distilled dionized H_2O	0	409.6 ± 37.0	388.4 ± 27.8
PBS	0.49	258.9 ± 27.0	316.0 ± 17.8
PBS + Urea	0.52	238.0 ± 34.4	257.6 ± 21.4
PUM	0.38	418.0 ± 27.6	399.6 ± 27.8
D5.45	0.08	495.4 ± 34.2	405.0 ± 17.8
D5.45 + Mg^{+2}	0.08	569.6 ± 30.3	342.6 ± 19.8
D5 (5% glucose)	0	224.0 ± 25.8	459.6 ± 29.6
.45 (.45% NaCl)	0.08	309.6 ± 11.8	344.4 ± 21.2
PUM + NaCl	0.52	193.0 ± 38.4	178.4 ± 24.6
PB + Urea	0.38	230.4 ± 27.4	216.6 ± 16.8

Table 3.

Adhesion of *Candida albicans* phenotypes to plastic

Strain	Phenotype	Mean No. *Candida*/mm² ± SD in:	
		PUM	D5.45
WO-1	white	156.0 ± 10.9	395.8 ± 31.0
	opaque	108.8 ± 8.9	210.5 ± 23.0
BL6	white	104.5 ± 30.6	274.9 ± 44.3
	opaque	196.0 ± 28.0	314.0 ± 39.8
6a	white	72.5 ± 24.6	543.3 ± 39.2
	opaque	88.0 ± 29.5	ND
	mycelial	10.3 ± 5.2	384.5 ± 41.4

Figure 1.

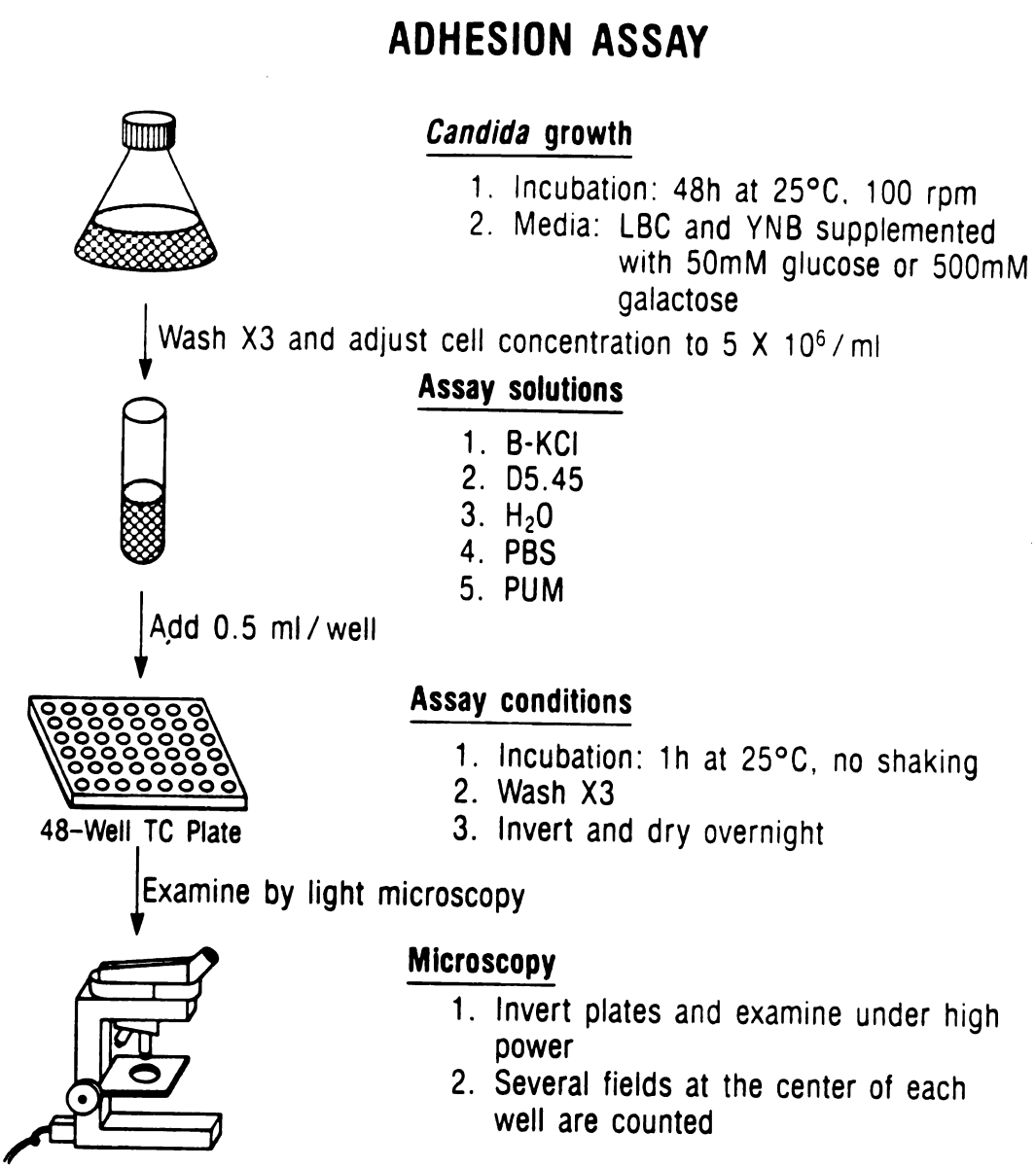

ADHESION ASSAY
Candida growth
1. Incubation: 48h at 25°C, 100 rpm
2. Media: LBC and YNB supplemented with 50mM glucose or 500mM galactose
Wash X3 and adjust cell concentration to 5 X 10⁶ / ml
Assay solutions
1. B-KCl
2. D5.45
3. H2O
4. PBS
5. PUM
Add 0.5 ml / well
48-Well TC Plate
Assay conditions
1. Incubation: 1h at 25°C, no shaking
2. Wash X3
3. Invert and dry overnight
Examine by light microscopy
Microscopy
1. Invert plates and examine under high power
2. Several fields at the center of each well are counted

Figure 2.

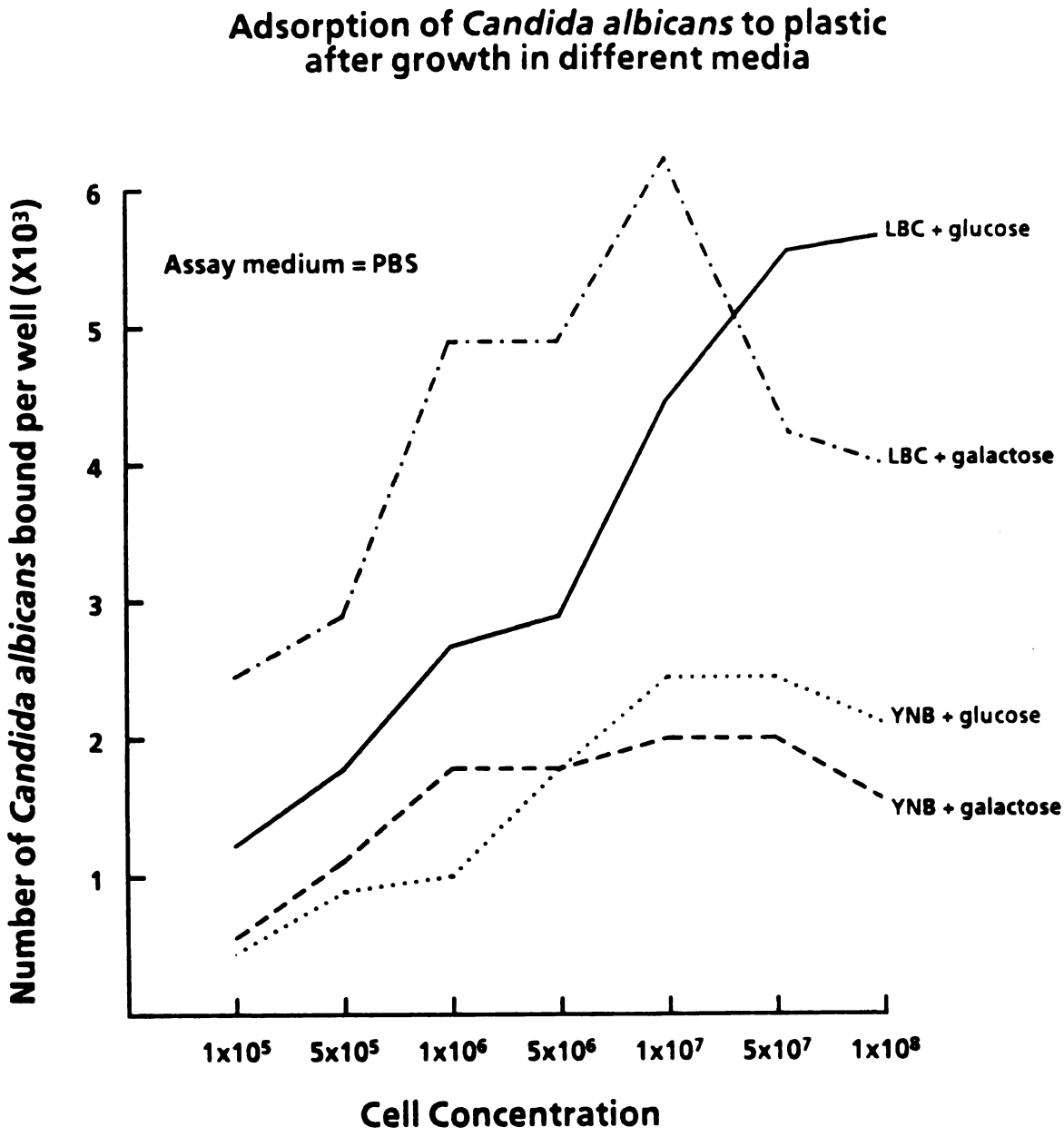

Adsorption of Candida albicans to plastic after growth in different media
Assay medium = PBS
LBC + glucose
LBC + galactose
YNB + glucose
YNB + galactose
Number of Candida albicans bound per well (X10³)
6
5
4
3
2
1
1x10⁵
5x10⁵
1x10⁶
5x10⁶
1x10⁷
5x10⁷
1x10⁸
Cell Concentration

Figure 3.

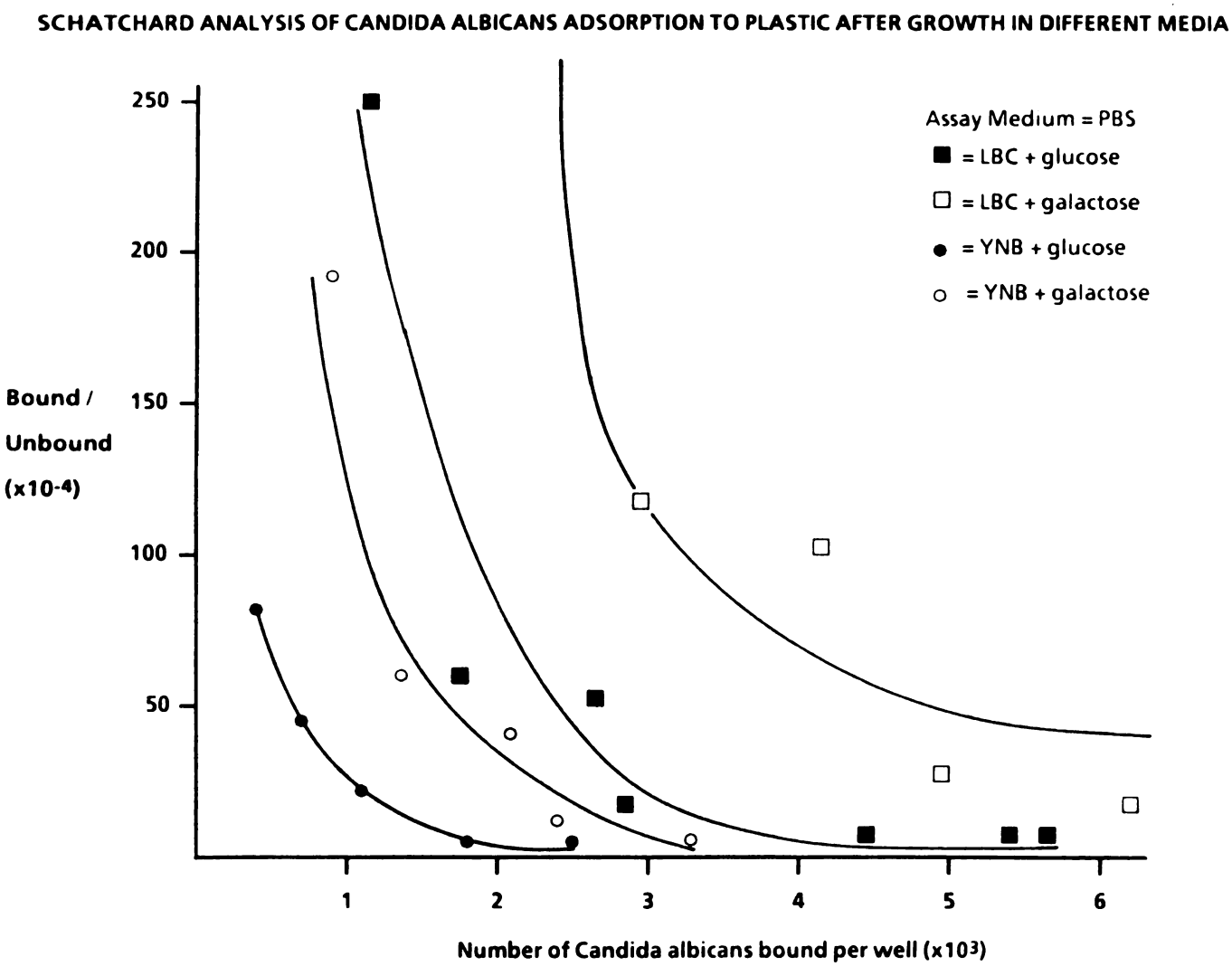

SCHATCHARD ANALYSIS OF CANDIDA ALBICANS ADSORPTION TO PLASTIC AFTER GROWTH IN DIFFERENT MEDIA
Assay Medium = PBS
■ = LBC + glucose
□ = LBC + galactose
● = YNB + glucose
○ = YNB + galactose
Bound / Unbound (x10-4)
250
200
150
100
50
1
2
3
4
5
6
Number of Candida albicans bound per well (x10³)

Figure 4. Effect of KCl concentration on adsorption of Candida albicans to plastic

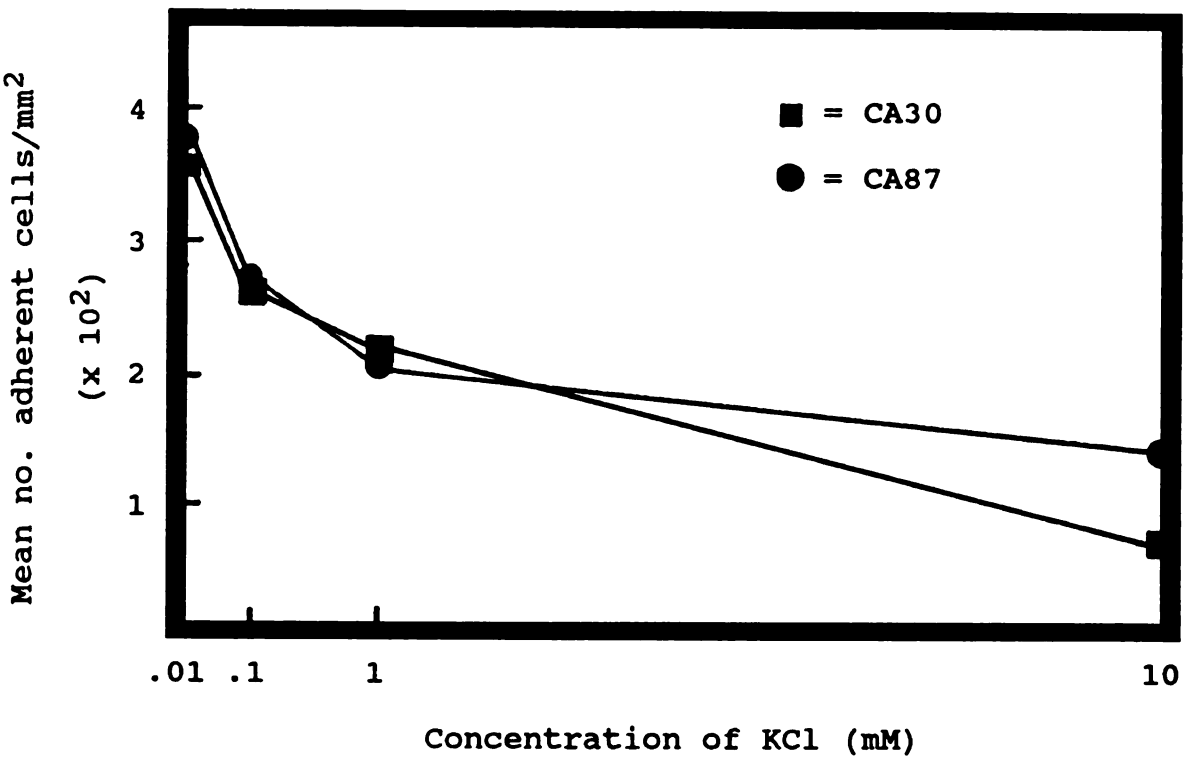


Figure 5. Effect of Mg^{+2} concentration on adsorption of Candida albicans to plastic

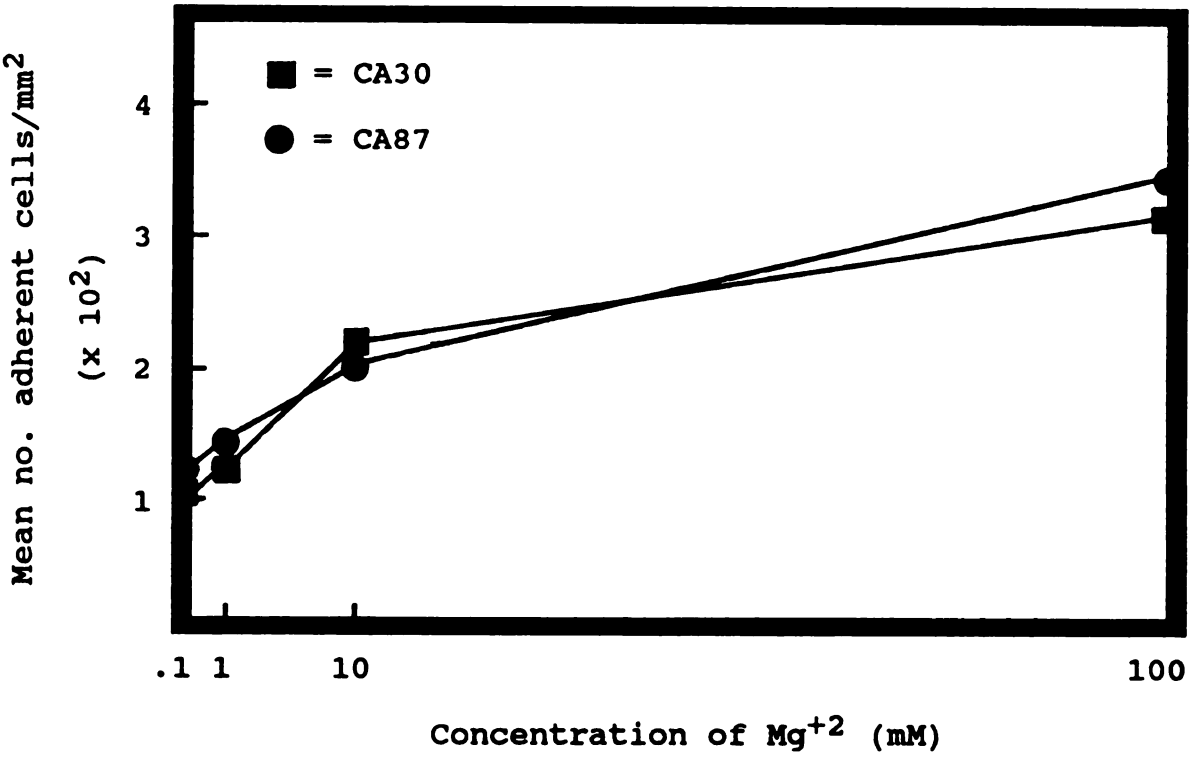
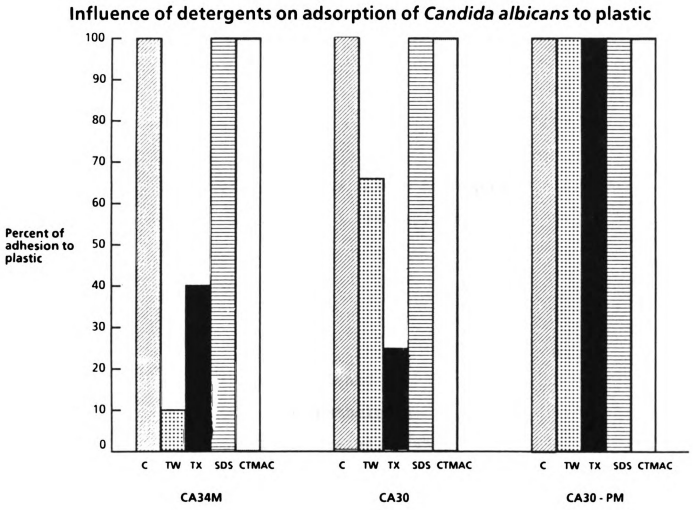


Figure 6.


Influence of detergents on adsorption of *Candida albicans* to plastic
Percent of adhesion to plastic
100
90
80
70
60
50
40
30
20
10
0
C TW TX SDS CTMAC
C TW TX SDS CTMAC
C TW TX SDS CTMAC
CA34M
CA30
CA30 - PM

AN ANAEROBIC CONTINUOUS-FLOW CULTURE MODEL OF INTERACTIONS BETWEEN INTESTINAL MICROFLORA AND *CANDIDA ALBICANS*

Michael J. Kennedy, Alvin L. Rogers,

and Robert J. Yancey, Jr.

Published in:

Mycopathologia

103: 125-134, 1988

An anaerobic continuous-flow culture model of interactions between intestinal microflora and *Candida albicans*

Michael J. Kennedy,[1,4,*] Alvin L. Rogers,[1,2,3] and Robert J. Yancey, Jr.[4]

Departments of [1]Botany and Plant Pathology, [2]Microbiology and Public Health, and [3]Medical Technology, Michigan State University, East Lansing, Michigan 48824 U.S.A., and [4]Microbiology and Nutrition Research, The Upjohn Company, Kalamazoo, Michigan 49001 U.S.A.

Running title: Models of interactions between intestinal bacteria and *Candida*

*Corresponding address: Microbiology and Nutrition Research, The Upjohn Company, 7922-190-MR, Kalamazoo, Michigan 49001 U.S.A.

SUMMARY

The finding by earlier workers that Escherichia coli suppressed the growth of Candida albicans in vitro or in gnotobiotic mice has led to numerous, erroneous conclusions regarding the identity of the organisms and mechanisms responsible for the suppression of Candida in the gut. This is due, in part, to the fact that nearly all studies to date have not reflected interactions as they occur in the intestinal tract. This paper describes a series of experiments that establish that an anaerobic continuous-flow (CF) culture model of the ecology of the large intestinal flora reproduces interactions between bacteria and Candida as they occur in the large intestine. This was determined in the following ways. (i) Bacterial counts in CF cultures of conventional mouse cecal flora or human fecal flora closely resembled that found in the mouse intestine and human feces. (ii) Dense layers of bacterial growth that formed on the glass walls of the CF culture vessels resembled bacterial populations that colonize intestinal mucosa. (iii) Total and individual levels of certain metabolic end-products of the predominant anaerobic bacterial flora present in CF cultures coincided with those found in the large intestine of conventional mice or human feces used to establish the CF cultures. (iv) C. albicans was eliminated from CF cultures of mouse cecal flora at a rate similar to that of

untreated experimental animals. (v) Contents of CF cultures fed to antibiotic-treated mice redressed several cecal abnormalities, and suppressed Candida populations to levels found in conventional animals. Thus, a number of complex ecological mechanisms were maintained in CF cultures which normally control Candida populations in the large intestine. It is suggested, therefore, that the CF culture model should help to further define the mechanisms which control C. albicans and other fungi in the intestinal tract, as well as define which components of the indigenous microflora are responsible for suppression of Candida in the gut.

INTRODUCTION

Historically, the study of interactions between the indigenous microflora and Candida albicans in the intestinal tract has been hampered by numerous problems. As one example, in an individual experimental animal, one is limited to studying such interactions at one time point, since, in most instances, the animal must be sacrificed to remove the specimen of interest. Furthermore, there is a limited range of experimental manipulations that are possible in the human or animal gut. To overcome these and other problems that come from using experimental animals, or human volunteers (23), there have been numerous attempts to study Candida-bacterial interactions in vitro (16,17,29,30). Unfortunately, however, these studies have also been beset with difficulties, and it is significant that none of these reflect interactions as they occur in the intestinal tract (9,10,22). Virtually all in vitro studies have demonstrated suppression of C. albicans by a single bacterial species (22,27), which is not representative of the 400 to 500 different bacterial species that normally inhabit the intestinal tract (7,25,33,37). Moreover, suppression of C. albicans using the former method has been shown to be dependent on culture conditions (29). Likewise, it has also been shown that certain intestinal bacterial species inhibitory to the growth of C. albicans

in vitro (16,17) were not inhibitory for *C. albicans* under normal *in vivo* conditions (1,5,22). One may conclude with some certainty, therefore, that there is little similarity between the inhibitory mechanisms for *C. albicans* in these simple *in vitro* systems and those found in the intestine, and that suppression of *C. albicans* by a single bacterial species cannot be expected to reflect interactions of a complex indigenous microflora (22).

Recently, an anaerobic continuous-flow (CF) culture model of the ecology of the large intestinal flora was developed that reproduces a number of bacterial interactions that occur in the large intestine of mice (14). For instance, it was found that mixed populations of mouse cecal bacteria in CF cultures were able to suppress "invader" bacterial populations (e.g., *Clostridium difficile* or *Escherichia coli*) to levels similar to those found after being fed to conventional mice (12,14, K.H. Wilson, J.N. Sheagren & R. Freter, 1984, Abstracts 84th Annual Meeting of the American Society for Microbiology), and that contents of CF cultures fed to germfree mice redressed several germfree abnormalities (e.g., cecal size and mucosal histology) (14). The finding that this model system can reproduce a variety of bacterial interactions that occur in the murine large intestine makes it likely that the underlying mechanisms that control the bacterial populations in anaerobic CF cultures are similar to those operating *in vivo* (12,14).

Because of this, and due to the shortcomings of the simple *in vitro* systems mentioned above, the present studies were designed to examine the use of an anaerobic CF culture model to study interactions between *C. albicans* and intestinal bacteria.

METHODS

Microorganisms

Bacteria for the inoculation of CF cultures or for recolonization of antibiotic-decontaminated mice were obtained from the cecal contents of normal mice, or the feces of a healthy volunteer who had not taken antibiotics for at least 24 months prior to the study. The bacterial suspensions were prepared in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) as described previously (22). Filtrates of cecal or CF culture contents were also prepared by a modification (22) of a previously described method (32). The isolate of *C. albicans* used was recovered from the blood of a patient with systemic candidosis (CA34). *Candida* cells were grown aerobically (without shaking) in Sabouraud dextrose broth (SDB; Difco Laboratories, Detroit, MI) for 18 h at 37°C.

Animals

Swiss Flow DUB/kr mice, 2 to 3 months old, were used in all experiments. These mice were originally obtained

from a closed colony at Eastern Michigan University, and a colony was subsequently established at The Upjohn Company. It is important to note the source of the mice used for the present studies because all major breeders supply "specific pathogen-free" (SPF) mice which originated from germfree stock and are unsuitable, therefore, for research on intestinal floral interactions (21). Such SPF animals lack strictly oxygen-sensitive anaerobes (13,14) and intestinal mucosa-associated populations (31), which predominate in the intestinal tracts of conventional animals (3,7,25,33,37), and are usually characterized by a flora containing environmental and handler microorganisms (13,14). To avoid these problems, mice were selected which contain a conventional intestinal microflora, and were not started from a germfree or SPF commercial stock.

Continuous-flow (CF) cultures

The CF culture apparatus described by Freter et al. (14) was used (Fig. 1). The growth medium was veal infusion broth (Difco) supplemented with 5 g l^{-1} yeast extract (Difco), 100 mg l^{-1} hemin (ICN Nutritional Biochemicals, Cleveland, OH), and 0.5 mg l^{-1} menadione (ICN Nutritional Biochemicals), and pumped into a growth tube containing a 7-ml culture at the rate of 1.17 ml h^{-1} by means of a Manostat Cassette Pump (Manostat, New York, NY).

This flow rate allowed fresh broth to be added to the

culture at a rate equivalent to displace 1/6 of the culture volume per h, and was chosen because previous studies had established that flow rates between 1/3 and 1/6 h^{-1} reacted similarly; but the slower flow rate conserved media, thereby facilitating long term experiments (14). Moreover, this flow rate simulated very closely the flow of intestinal contents through the mouse cecum (14). The 7-ml culture was contained in a round glass tube (approximately 12-mm inner diameter), and was stirred at 500 rpm by means of a Teflon-covered magnetic stirring "flea" (10-mm length, 3-mm diameter). Because of the small size of the stirring bar, this stirring action was adequate to cause a barely noticeable movement of the medium at the culture surface. The entire apparatus was maintained in an anaerobic chamber at 37°C. The atmosphere in the chamber consisted of 5% CO_2, 10% H_2, and 85% N_2, and was maintained at less than 5 ppm O_2 by means of a coated palladium catalyst (2). The oxygen level was monitored with a Trace Oxygen Analyzer (2). Inoculation of CF cultures was carried out in the following manner. A freshly killed mouse or human feces was placed immediately into the anaerobic chamber. Dissection of the mouse was performed by reflecting the skin coverings and abdominal walls in layers to avoid contamination of the viscera. The cecum was then aseptically removed, and part of the cecal contents and a small piece of the cecal wall were introduced into the growth tube. For human feces, samples

were well mixed according to previously described methods (15), and part of the feces was similarly introduced into a separate CF culture growth tube. CF cultures were allowed to equilibrate for a three-week period before being used in experiments.

Animal preparation, inoculation, and recolonization experiments

Mice were housed in sterile, covered plastic cages and supplied with sterile Purina mouse chow and sterile distilled water containing vancomycin (500 $\mu g\ ml^{-1}$), ampicillin (1 $mg\ ml^{-1}$), and gentamicin (100 $\mu g\ ml^{-1}$) (VAG) ad libitum for 5 days (19,20,22). Control animals were housed identically but without the addition of VAG to the drinking water. After decontamination, mice were transferred to sterile cages as described previously (19,20).

To test the ability of indigenous cecal bacteria to protect against C. albicans colonization, cecal or CF culture homogenates (or filtrates as controls) were administered to antibiotic-decontaminated mice by both oral and rectal routes once daily for 5 consecutive days, beginning 5 days after stopping antibiotic treatment. For oral administration, mice were lightly anesthetized with methoxyflurane (Pittman-Moore Co., Washington Crossing, NJ), and cecal homogenates or filtrates were administered by carefully inserting a 5-cm 18-gauge plastic catheter

through the mouth into the stomach and injecting 1 ml of the material. For rectal administration, the same catheter was placed approximately 5 mm into the rectum, the skin was pinched tightly around the catheter, and 2 ml of the material was injected. The latter dose has been shown to be an amount adequate to reach the cecum (40). On the day after the last administration of cecal homogenates or filtrates, the animals were challenged intragastrically (as above) with 0.5 ml of Sabouraud dextrose broth containing 10^7 CFU of C. albicans.

Enumeration of C. albicans

Counts of viable C. albicans were performed on cecal or CF culture contents at various intervals after administration of C. albicans. Mice were killed by cervical dislocation, their abdomens were soaked with 70% ethanol, and skin coverings and abdominal walls were reflected in layers to avoid contamination of the viscera. Ceca were aseptically excised and placed into separate glass tissue homogenizers, each containing 2 ml of sterile SDB. Organs were homogenized, and serial 10-fold dilutions of organ homogenates or CF cultures were plated on Sabouraud dextrose agar supplemented with vancomycin (7.5 $\mu g\ ml^{-1}$) and ampicillin (100 $\mu g\ ml^{-1}$) for the enumeration of C. albicans. Plates were incubated aerobically for 48 h at 37°C to determine the number of colonies per organ.

Enumeration of indigenous cecal bacteria

Population levels of indigenous bacteria colonizing the CF cultures were determined in the anaerobic chamber. Samples were removed aseptically from the CF culture, and serial 10-fold dilutions were made in tryptic soy broth (Difco), supplemented with 0.04% Na_2 CO_3, to compensate for the 5% CO_2 in the anaerobic chamber (39), and 0.1 ml was plated on blood agar, MacConkey agar (Difco), and modified AII agar (21,22) supplemented with 0.05% maltose (Rolf Freter, personal communication) without palladium chloride (28). Mammary powder (ICN Nutritional Biochemicals) was also substituted for placenta powder in the AII medium (R. Freter, personal communication). The resulting medium yielded colony counts of strictly anaerobic bacteria equal to the "old" AII agar (21). All manipulations were performed in the anaerobic chamber, and the media and other materials were placed in the chamber at least 48 h prior to use.

Modified AII agar plates were incubated at 37°C in the anaerobic chamber for at least 5 days. Numbers of facultative anaerobic bacteria and Enterobacteriaceae were determined by incubating for 48 h at 37°C one set each of MacConkey and blood agar plates aerobically and an identical set of plates anaerobically for 48 h at 37°C.

Volatile fatty acid (VFA) analyses

The concentrations of VFAs in the cecal contents,

feces, or CF cultures were determined chromatographically (6), with 2-methylpentanoic acid as an internal standard (24), as described previously (22).

RESULTS

In the first study, the similarity of the bacterial flora from CF cultures and mouse cecal contents or human feces was determined by performing selective plate counts. These results are summarized in Table 1. Facultative and strictly anaerobic bacteria were found in almost identical numbers in CF cultures and in the ceca of mice or human feces. Likewise, the total numbers of Enterobacteriaceae found in CF cultures of human fecal flora and in human feces were also nearly identical, while the numbers of Enterobacteriaceae were found to be slightly higher in CF cultures of mouse cecal flora compared to those found in the mouse cecum. Therefore, both the total number of bacteria and the populations of various types of intestinal bacteria were very similar in CF cultures and their *in vivo* counterparts. Furthermore, gram stains of cecal contents from CF cultures, mouse ceca, and human feces were also remarkably similar. In all instances, the flora was characterized by a predominance of gram-negative rods. Another similarity between these systems was that dense layers of bacterial growth formed on the glass walls of the CF culture vessels, analogous to

bacterial populations that colonize intestinal mucosa. Figure 2 shows a bacterial layer that formed on the CF culture vessel containing mouse cecal flora, and is typical of the growth on the glass wall after equilibration with the entire complex intestinal flora.

Table 2 summarizes the results of a series of experiments in which VFAs present in CF cultures, mouse ceca, and human feces were determined. The VFAs are regulated by the predominate anaerobic flora present in an ecosystem [15]. Both the total and individual levels of VFAs present in CF cultures coincide with the quantity of VFAs found in the ceca of the conventional mice used, as well as with those found in the feces used to establish the CF cultures of human fecal flora. The only significant differences found were for acetic and isovaleric acid ($P < 0.05$), which were lower and higher, respectively, in CF cultures of mouse cecal flora. The patterns of VFA levels were, nevertheless, consistent for all experiments, and strongly suggest that the bacterial floras of the CF cultures were very similar to those found *in vivo*.

Figure 3 illustrates another critical test to determine the similarity of interactions between *C. albicans* and the intestinal microflora in these systems, and whether CF cultures would serve as good models of such interactions. *Candida* cells were inoculated into mice or CF cultures of mouse cecal flora, and the numbers of

viable C. albicans were determined at various intervals thereafter. The data points represent the logarithmic means of three separate experiments. As may be seen, the elimination of C. albicans from the mouse cecum occurred at a rate similar to that observed when C. albicans was inoculated into a CF culture of mouse cecal flora, thus indicating that the CF cultures reliably reproduced the populations of C. albicans as they occurred in mice under normal in vivo conditions.

As a final means of testing the ability of CF cultures to maintain the indigenous bacterial flora, and, therefore, a number of interactions as they occur in vivo, the material from CF cultures of mouse cecal flora was fed to antibiotic-decontaminated mice to determine if they could redress several abnormalities that occur when the bacterial flora is eliminated (11,35,36), including the ability to resist colonization by C. albicans. For these studies (Table 3), mice were given a combination of antibiotics (VAG) designed to eliminate most of their intestinal flora, and subsequently they were given oral and rectal injections of either CF culture material, cecal homogenates, or filtrates of cecal homogenates. These animals, as well as appropriate control animals, were then inoculated with 10^7 C. albicans, and cultured 24 h postinoculation to determine the levels of Candida in their ceca. Antibiotic-treated animals challenged with C. albicans had high levels of C. albicans colonizing their

ceca, as did animals given antibiotics followed by cecal filtrates. Control animals not given antibiotics, and antibiotic-treated mice recolonized with the indigenous cecal microflora, in contrast, had significantly lower cecal populations of C. albicans ($P < 0.001$). Likewise, animals that had been given CF culture material also had levels of Candida colonizing their ceca that were significantly lower than mice given antibiotics only ($P < 0.001$).

The effects of antibiotics and recolonization on the indigenous cecal microflora and certain cecal characteristics are summarized in Table 4. Animals treated with antibiotics and animals treated with antibiotics followed by cecal filtrates had several cecal abnormalities. Gram stains, for instance, showed these animals to contain few bacteria (mostly gram-positive cocci), with a complete disappearance of the predominant gram-negative and fusiform-shaped rods. In contrast, antibiotic-treated mice, which had been recolonized with cecal bacteria isolated from the ceca of mice or with CF cultures of mouse cecal flora by oral and rectal injections, had bacterial floras that were similar to those found in conventional animals. Furthermore, dissection of test and control animals revealed other differences in cecal characteristics according to the experimental group examined. Animals that possessed a complex bacterial flora (i.e., normal mice, or mice

recolonized with mouse cecal contents or CF cultures of mouse cecal bacteria) had small ceca (approximately 1% to 2% of their total body weight) which contained thick, pasty contents. Mice given antibiotics to eliminate the indigenous microflora, in contrast, had enlarged ceca (approximately 5% to 8% of their total body weight) with very watery contents, a trait attributable to mice lacking a complex ("normal") intestinal microflora (8,11,35,36,38).

DISCUSSION

The results presented in this paper strongly support the hypothesis that the major ecological mechanisms that control and maintain a balance among bacterial populations of the large intestine are reproduced in CF cultures (12,14). The ability of CF cultures to maintain a bacterial flora that closely resembled that found in the mouse large intestine, for instance, is probably the most convincing of these data. Furthermore, thick layers of bacteria formed on the walls of the CF cultures, and these resembled mucosa-associated populations found in the intestine (14,34). It is known that indigenous bacteria of the large intestine occur in thick layers in the mucosa (34), presumably because the bacteria adhere to each other rather than to the epithelium itself (14). Scrapings of the wall layer of CF cultures revealed that the bacteria

were adhering to each other. This similarity, therefore, constitutes additional supporting evidence that CF cultures reproduce a number of bacterial interactions that occur in the large intestine (12,14), and is consistent for both mouse and human intestinal flora. If the mucosal flora is disrupted, as with antibiotic therapy, the intestinal microflora undergoes a significant metabolic change (8,35). Likewise, if the formation of these layers is prevented in the CF culture, the resulting flora no longer resembles the intestinal flora in its function (14). As noted by Freter *et al.* (14), one or a few bacterial species usually will overgrow all others when a clinical specimen is removed from the body and allowed to multiply in artificial nonselective culture. Because the major bacterial groups were present in similar numbers in CF cultures and the ecosystems from which they were obtained, it is likely that the ecological control mechanisms operating *in vitro* were the same, or at least very similar to, those controlling the microflora *in vivo*.

This interpretation was further supported by the findings that the qualitative and quantitative composition of various VFAs present in CF cultures closely resembled that in the mouse cecum and human feces. Since these acids are characteristic metabolic end products of the predominant anaerobic bacteria that colonize the large intestine (15), it may be concluded that the CF cultures were able to support the major metabolic activities of the

predominate intestinal anaerobes. Therefore, because it is unlikely that two different sets of mechanisms would bring about similar equilibria among a complex microflora (14), such as those studied here, one may conclude with some certainty that the predominant microbial interactions were reproduced in CF cultures as they occur in the large intestine. Indeed, when the survival and passage of C. albicans through CF cultures of mouse cecal flora and the mouse cecum proper were compared, Candida cells were found to be eliminated at a similar rate.

A final stringent test of the ability of CF cultures to propagate all of the numerous bacterial species of the cecal flora came from experiments showing that CF culture material could redress several cecal abnormalities of antibiotic-treated mice (11,35,36,38). For example, when CF culture material was fed to antibiotic-treated mice, a complete reduction of the enlarged cecum to the normal size took place. Likewise, a bacterial flora resembling that of conventional mice not given antibiotics was confirmed by observing gram-stained smears of the cecal contents from normal and antibiotic-treated mice reconventionalized with CF culture suspensions. Moreover, such reconventionalized mice were able to resist colonization by C. albicans, again suggesting that at least those organisms responsible for the suppression of Candida in the gut were maintained in this culture system.

It is significant to note that a complete reduction

of the enlarged cecum to normal size and a reduction of the intestinal population of Candida to levels found in conventional animals have never been fully achieved in germfree or antibiotic-treated animals following the implantation of one or a few bacterial species (4,5,16,17,26). Clark (5), for instance, showed that C. albicans grew unchecked for several weeks in the GI tracts of gnotobiotic mice containing an intestinal flora of Bacteroides sp., Lactobacillus sp., Streptococcus faecalis, Streptococcus lactis, and Escherichia coli. Nevertheless, studies by Freter and coworkers (11,38) indicated that a complex intestinal flora, comprised predominantly of strict oxygen-sensitive anaerobes, was required to redress several ceca abnormalities of germfree mice. Furthermore, it was also found that a complex intestinal flora was required to maintain a balance among several bacterial populations similar to that found in conventional animals (14). In fact, close approximation of these parameters to normal values required the implantation of whole cecal contents or no less than 95 metabolically distinct strict anaerobes (11). The present findings that whole cecal contents or CF cultures of the cecal microflora were able to convert antibiotic-treated mice to the normal state and to reduce Candida populations to levels similar to those found in conventional animals, then, are consistent with the view that a complex and diverse series of interactions is responsible for the

control of Candida in the gut. Consequently, the CF culture model presented in this paper offers an excellent and accessible way to study Candida-bacterial interactions in vitro. This model is currently being used to address the question of the nature of the mechanisms that control C. albicans in CF cultures of intestinal microflora.

REFERENCES

1. Andremont, A., Raibaud, P. & Trancrede, C. 1983. Effect of erythromycin on microbial antagonism: a study in gnotobiotic mice associated with a human fecal flora. Journal of Infectious Diseases, 148, 579-587.

2. Aranki, A. & Freter, R. 1972. Use of anaerobic glove boxes for the cultivation of strictly anaerobic bacteria. American Journal of Clinical Nutrition, 25, 1329-1334.

3. Aranki, A., Syed, S.A., Kenney, E.B. & Freter, R. 1969. Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box procedure. Applied Microbiology, 27, 568-576.

4. Balish, E. & Phillips, A.W. 1966. Growth and virulence of Candida albicans after oral inoculation in the chick with a monoflora of either Escherichia coli or Streptococcus faecalis. Journal of Bacteriology, 91,

1744-1749.

5. Clark, J.D. 1971. Influence of antibiotics or certain intestinal bacteria on orally administered Candida albicans in germ-free and conventional mice. Infection and Immunity, 4, 731-737.

6. Cottyn, B.G. & Boucque, C.F. 1968. Rapid method for the gas-chromatographic determination of volatile fatty acids in rumen fluid. Journal of Agricultural Food Chemistry, 16, 105-107.

7. Dubos, R., Schaedler, R.W., Costello, R. & Hoet, P. 1965. Indigenous, normal, and autochothonous flora of the gastrointestinal tract. Journal of Experimental Medicine, 122, 67-76.

8. Dubos, R., Schaedler, R.W. & Stevens, M. 1963. The effect of antibacterial drugs on the fecal flora of mice. Journal of Experimental Medicine, 117, 231-243.

9. Freter, R. 1976. Factors controlling the composition of the intestinal microflora. In: H.W. Stiles, W.J. Loesche, and T.C. O'Brien (Ed.), Proceedings: microbial aspects of dental caries (a special supplement to Microbiology Abstracts), vol. 1, pp 109-120. Information Retrieval Inc., Washington, D.C.

10. Freter, R. 1983. Mechanisms that control the microflora in the large intestine. In: D.J. Hentges (Ed.), Human intestinal microflora in health and disease, p. 33-54. Academic Press, Inc., New York.

11. Freter, R. & Abrams, G.D. 1972. Function of various intestinal bacteria in converting germ-free mice to the normal state. Infection and Immunity, 6, 119-126.

12. Freter, R., Brickner, H., Botney, M., Cleven, D. & Aranki A. 1983. Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora. Infection and Immunity, 39, 676-685.

13. Freter, R., Brickner, H., Fekete, J., O'Brien, P.C.M. & Vickerman, M.M. 1979. Testing of host-vector systems in mice. Recombinant DNA Technical Bulletin, 2, 68-76.

14. Freter, R., Stauffer, E., Cleven, D., Holdeman, L.V. & Moore, W.E.C. 1983. Continuous-flow cultures as in vitro models of the ecology of large intestinal flora. Infection and Immunity, 39, 666-675.

15. Holdeman, L.V., Cato, E.P. & Moore, W.E.C. , eds. 1977. Anaerobe Laboratory Manual, 4th ed. Virgina Polytechnic Institute and State University, Blacksburg,

Virginia.

16. Hummel, R.P., Maley, M.P., Miskell, P.W. & Altemeier, W.A. 1975. Suppression of Candida albicans by Escherichia coli. Journal of Trauma, 15, 413-418.

17. Hummel, R.P., Oestreicher, E.J., Meley, M.P. & MacMillan, B.G. 1973. Inhibition of Candida albicans by Escherchia coli in vitro and in the germfree mouse. Journal of Surgical Research, 15, 53-58.

18. Iwai, H., Ishihara, Y., Yamanaka, J. & Ito, T. 1973. Effects of bacteria on cecal size and transit rate of intestinal contents in mice. The Japanese Journal of Experimental Medicine, 43, 297-305.

19. Kennedy, M.J., Bajwa, P.S. & Volz, P.A. 1982. Gastrointestinal inoculation of Sporothrix schenckii in mice. Mycopathologia, 78, 141-143.

20. Kennedy, M.J. & Volz, P.A. 1983. Dissemination of yeasts after gastrointestinal inoculation in antibiotic-treated mice. Sabouraudia, 21, 27-33.

21. Kennedy, M.J. & Volz, P.A. 1985. Effect of various antibiotics on gastrointestinal colonization and dissemination by Candida albicans. Sabouraudia: Journal

of Medical and Veterinary Mycology, 23, 265-273.

22. Kennedy, M.J. & Volz, P.A. 1985. Ecology of Candida albicans gut colonization: Inhibition of Candida adhesion, colonization, and dissemination from the gastrointestinal tract by bacterial antagonism. Infection and Immunity, 49, 654-663.

23. Krause, W., Matheis, H. & Wulf, K. 1969. Fungaemia and funguria after oral administration of Candida albicans. Lancet, 1, 598-599.

24. Mayhew, J.W. & Gorbach, S.L. 1977. Internal standards for the gas chromatographic analysis of metabolic end products from anaerobic bacteria. Applied and Environmental Microbiology, 33, 1002-1003.

25. Moore, W.E.C. & Holdeman, L.V. 1974. The normal flora of 20 Japanese-Hawaiians. Applied Microbiology, 27, 961-979.

26. Nishikawa, T., Hatano, H., Ohnishi, N., Susaki, S. & Omura, T. 1969. Establishment of Candida albicans in the alimentary tract of the germfree mice and antagonism with Escherichia coli after oral inoculation. Japan Microbiology, 13, 263-276.

27. Odds, F.C. 1979. Candida and Candidosis, University Park Press, Baltimore.

28. Owens, D.R., Rolfe, R.D. & Hentges, D.J. 1976. Effectiveness of palladium chloride for the isolation of anaerobes. Journal of Clinical Microbiology, 3, 854-861.

29. Ozawa, K., Jabu-Uchi, K., Yamanaka, K., Jamachita, Y., Ueba, K. & Micwatani, T. 1979. Antagonistic effects of Bacillus nato and Streptococcus facealis on growth of Candida albicans. Microbiology and Immunology, 23, 1147-1156.

30. Paine, T.F., Jr. 1958. The inhibitory activity of bacteria on Candida growth. Antibiotics and Chemotherapy, 8, 273-281.

31. Phillips, M.W. & Lee, A. 1984. Microbial colonization of rat colonic mucosa following intestinal pertubation. Microbial Ecology, 10, 79-88.

32. Rifkin, G.D., Fekety, F.R., Silva, J. & Sack, R.B. 1977. Antibiotic-induced colitis: implication of a toxin neutralized by Clostridium sordellii anti-toxin. Lancet, ii, 1103-1106.

33. Savage, D.C. 1977. Microbial ecology of the

gastrointestinal tract. Annual Review of Microbiology, 31, 107-133.

34. Savage, D.C. 1980. Adherence of normal flora to mucosal surfaces. In: E.H. Beachey (Ed) Bacterial Adherence, pp. 33-59. Chapman & Hall, London.

35. Savage, D.C. & Dubos, R. 1968. Alterations in the mouse cecum and its flora produced by antibacterial drugs. Journal of Experimental Medicine, 128, 97-110.

36. Savage, D.C. & McAllister, J.S. 1971. Cecal enlargement and microbial flora of suckling mice given antibacterial drugs. Infection and Immunity, 3, 342-349.

37. Schaedler, R.W., Dubos, R. & Costello, R. 1965. The development of the bacterial flora in the gastrointestinal tract of mice. Journal of Experimental Medicine, 122, 59-65.

38. Syed, S.A., Abrams, G.D. & Freter, R. 1970. Efficiency of various intestinal bacteria in assuming normal functions of enteric flora after association with germfree mice. Infection and Immunity, 2, 376-386.

39. Wilson, K.H., Kennedy, M.J. & Fekety, F.R. 1982. Use of sodium taurocholate to enhance spore recovery on a

selective medium for Clostridium difficile. Journal of Clinical Microbiology, 15, 443-446.

40. Wilson, K.H., Silva, J. & Fekety, F.R. 1981. Suppression of Clostridium difficile by normal hamster cecal flora and prevention of antibiotic-associated colitis. Infection and Immunity, 34, 626-628.

Table 1. Population levels of intestinal bacteria in the ceca of test animals, human feces, and anaerobic continuous-flow (CF) cultures.

Experimental group (n)	Log_{10} mean no (± SD) per g (wet wt.) or ml		
	Facultative bacteria	Enterobacteriaceae	Strictly anaerobic bacteria
Mouse cecal flora (10)	8.4 ± 0.4	4.9 ± 0.2	9.9 ± 0.1
CF culture of mouse cecal flora (4)	8.3 ± 0.3	6.3 ± 0.3	9.9 ± 0.2
Human fecal flora (2)	8.5 ± 0.9	6.5 ± 0.8	8.9 ± 0.5
CF culture of human fecal flora (2)	8.1 ± 0.8	6.8 ± 0.7	8.7 ± 0.4

Table 2. Concentrations of volatile fatty acids in test animals, human feces, and in anaerobic continuous-flow (CF) cultures.

Experimental Group (n)	Volatile fatty acid concentration (mM) of contents ± SD					
	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric
Mouse cecal contents (5)	116.1 ± 19.2	20.4 ± 4.5	4.5 ± 0.8	20.8 ± 5.7	3.8 ± 0.8	1.3 ± 0.6
CF culture of mouse cecal flora (5)	79.3 ± 2.9	19.1 ± 6.0	3.9 ± 1.2	21.8 ± 3.4	7.4 ± 3.1	0.6 ± 0.8
Human feces (2)	77.7 ± 12.6	29.9 ± 4.6	4.1 ± 0.8	30.4 ± 6.3	9.2 ± 0.7	1.5 ± 0.1
CF culture of human fecal flora (2)	70.5 ± 15.1	21.3 ± 4.9	5.4 ± 1.1	29.5 ± 16.4	8.0 ± 1.7	1.7 ± 0.6

Table 3. Cecal population levels of *Candida albicans* (CA34) in reconventionalized and control animals.

Antibiotic treatment	Log_{10} mean no. ± SD of *Candida* per g[b]
Antibiotics only (VAG)[a]	5.7 ± 1.2
Antibiotics, followed by cecal filtrates	5.8 ± 0.9
Antibiotics, followed by cecal homogenates	1.8 ± 0.9
Antibiotics, followed by continuous-flow culture material	2.3 ± 1.5
None	2.6 ± 1.6

[a] Animals received vancomycin, ampicillin, and gentamicin (VAG) in the drinking water as described in the text.
[b] The values represent the means and standard deviations from 5 to 10 mice per group cultured for *C. albicans* at 24 h postinoculation.

Table 4. Cecal characteristics of antibiotic-treated, control, and reconventionalized animals.

Animal treatment[a]	Gram stain[b]	Predominant bacterial organisms[c]	Consistency of cecal contents	Cecal size[d]
Antibiotics only (VAG)	–	Gm- cocci, and small Gm- rods	Soft	5 – 8%
Antibiotics, followed by cecal filtrates	–	Gm- cocci, and small Gm- rods	Soft	5 – 8%
Antibiotics, followed by cecal homogenates	+	Large, fusiform-shaped Gm- rods	Thick and pasty	1 – 2%
Antibiotics, followed by continuous-flow culture material	+	Large, fusiform-shaped Gm- rods	Thick and pasty	1 – 2%
None	+	Large, fusiform-shaped Gm- rods	Thick and pasty	1 – 2%

[a] Animals received vancomycin, ampicillin, and gentamicin (VAG) in the drinking water was described in the text.
[b] Gram strins: – = abnormal; + = normal compared to normal, untreated animals.
[c] Predominant organisms seen on gram stained smears.
[d] Percentage of total body weight.

Figure 1. The continuous-flow culture apparatus.

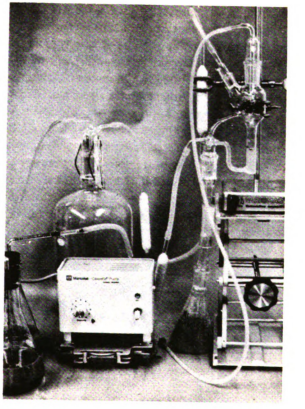
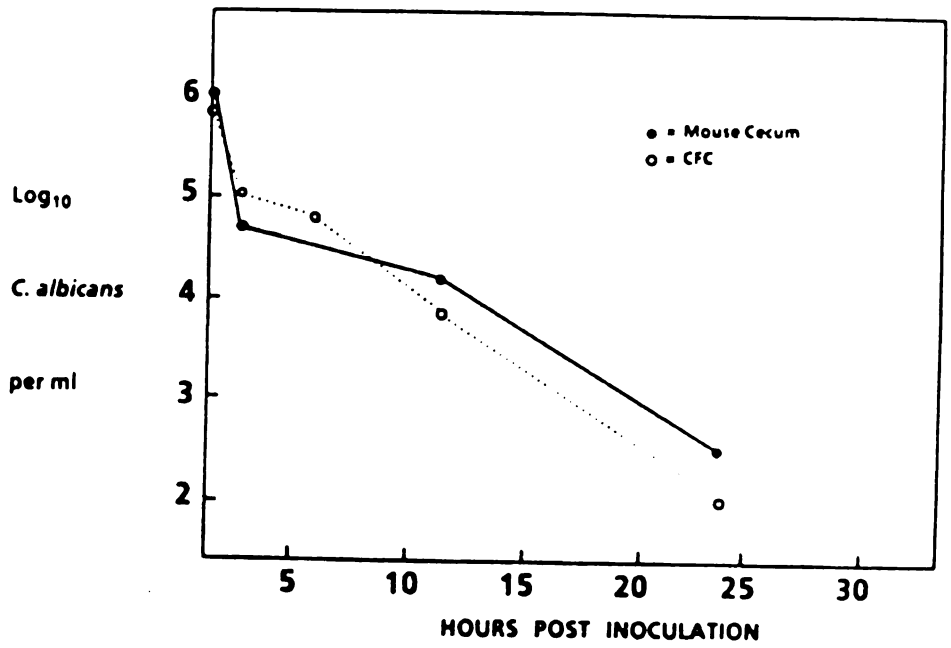


Figure 2. A continuous-flow culture growth tube showing dense layers of bacterial growth formed on the wall that resemble bacterial populations that colonize intestinal mucosa.

Figure 3. Elimination of C. albicans CA34 from continuous-flow culture of mouse cecal flora and mouse cecum. The values represent the means from 4 to 10 samples per data point.



MECHANISMS THAT CONTROL *CANDIDA ALBICANS* POPULATIONS IN CONTINUOUS-FLOW CULTURE MODELS OF INTESTINAL MICROFLORA AND IN THE LARGE INTESTINE

Michael J. Kennedy, Alvin L. Rogers,

and Robert J. Yancey, Jr.

Submitted for publication to:

Infection and Immunity

October 10, 1988

Mechanisms that control *Candida albicans* populations in continuous-flow culture models of intestinal microflora and in the large intestine

Michael J. Kennedy[1,4*], Alvin L. Rogers[1,2,3], and Robert J. Yancey, Jr.[4]

Departments of [1]Botany and Plant Pathology, [2]Microbiology and Public Health, and [3]Medical Technology, Michigan State University, East Lansing, Michigan 48824, and [4]The Upjohn Company, Kalamazoo, Michigan 49001

Running title: Control of *Candida* by intestinal microflora

*Correspondence address:

The Upjohn Company
Microbiology and Nutrition Research
7922-190-MR
Kalamazoo, Michigan 49001

ABSTRACT

It has been well established that the intestinal microflora controls Candida albicans populations in the gastrointestinal (GI) tract. However, because most early studies did not reflect interactions as they occur in vivo, several erroneous conclusions have been made regarding the identity of the organisms and mechanisms responsible for the suppression of Candida in the gut. This paper describes the suppression of C. albicans in a continuous-flow (CF) culture model of the large intestinal microflora and in the mouse large intestine by a complex indigenous microbiota. When inoculated into CF cultures of established mouse cecal flora, C. albicans was rapidly eliminated at a rate greater than that of the system. In contrast, when C. albicans was inoculated into CF cultures of established human fecal flora or simultaneously or before establishment of mouse cecal flora, C. albicans was removed with the flow rate of the system. These results suggest that C. albicans can develop resistance/tolerance to some or all inhibitory mechanisms in the CF culture. Growth rates of C. albicans in CF cultures or in sterile filtrates of CF cultures incubated anaerobically were considerably slower than in CF culture medium incubated aerobically. Anaerobic condition in CF culture medium, however, was only partially inhibitory to growth of C. albicans, indicating that other mechanisms were also operating to suppress the growth of C. albicans. The

inhibitory effect on Candida, moreover, could not be abolished by adding carbon or nitrogen sources, vitamins, or all three to CF culture filtrates and incubating the system (under varying pHs) aerobically. Apparently, suppression of C. albicans was not due solely to depletion of nutrients and anaerobiosis, but may instead be due to a combination of these two factors in addition to the production of inhibitory substances by intestinal bacteria. The production of the short-chain fatty acids acetic acid and butyric acid by intestinal anaerobes were shown to be inhibitory to C. albicans growth. In addition, mucosal association was found to be important for long term colonization of the cecum of antibiotic-treated animals as the flora reestablished. It is suggested that the indigenous intestinal microflora controls C. albicans populations collectively by several mechanisms, but that Candida cells can become tolerant to these mechanisms and can survive at a low levels in the gut probably by associating with GI mucosal surfaces.

INTRODUCTION

Colonization of the gastrointestinal (GI) tract by Candida albicans may play an important role in diseases of humans since the GI tract is considered the proximate source of infection in recurrent vaginitis (23,26) and systemic candidosis (22,24,30-32). Although the determinants of GI colonization by C. albicans are not completely defined (20,21), it is known that certain members of the indigenous microflora suppress the growth of C. albicans within the gut (4-7,11-13,18-21,25,28-32). Nevertheless, because the intestinal microflora is able to regulate Candida populations in the gut and control the spread of C. albicans to other body sites (18-20), elucidation of the inhibitory mechanisms should lead to a better understanding of the ecology of Candida gut colonization. In addition, such knowledge may also lead to ways to manipulate the GI environment for the specific purpose of inhibiting fungal growth (15).

Previous attempts to determine the mechanisms by which intestinal bacteria inhibit C. albicans (11-13,29) have been beset with methodologic difficulties (see 17). As commented on by Freter (8-10), there are several reasons why such studies were "doomed to failure". First among these is that most in vitro studies have relied on simple static cultures of a diflora of C. albicans and a single bacterial species (4,11-13,29), which do not reflect interactions as they normally occur in the

intestinal tract by a complex indigenous microflora (8,20). Moreover, simple dicultures can be manipulated to give varying results depending on, for example, the culture medium used (28). Moreover, several of these studies have shown that C. albicans was strongly inhibited by certain bacterial species in vitro (4,11,12,25), whereas others have shown that these same organisms had no effect on C. albicans growth in the mouse intestine (6,19). It is clear from the above list that in vitro model systems will have to closely simulate the processes occurring in conventional animals or humans if meaningful results are to be gained from studies of bacterial-Candida interactions (10).

An anaerobic continuous-flow (CF) culture model of the ecology of the mouse large intestinal flora was recently developed which meets these stringent requirements, since it reproduces a number of microbial interactions and maintains the indigenous microflora in vitro as it occurs in vivo (9,10). In a previous paper (17) evidence was presented which supports the findings that populations of intestinal bacteria in CF cultures of mouse cecal flora resemble those in the mouse intestine, and offers additional evidence that ecological mechanisms and environmental conditions prevailing in both systems are very similar. Furthermore, it was shown that interactions between C. albicans and the intestinal microflora in CF cultures and in the mouse large intestine

were very similar, since population levels of C. albicans and elimination rates were almost indistinguishable. That study, however, did not address the question of what mechanisms were responsible for the suppression of C. albicans by intestinal bacteria. This paper, therefore, describes the mechanisms which regulate Candida populations in this culture system, and describes the suppression of C. albicans not by a single bacterial species but by a complex intestinal microflora.

MATERIALS AND METHODS

Microorganisms. Bacteria for the inoculation of CF cultures were obtained from the cecal contents of Swiss Flow DUB/kr mice (19) or the feces of a healthy male volunteer on a Western diet who had not taken antibiotics for at least 24 months prior to the study (17). The bacterial suspensions were prepared in a Coy Anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) as described previously (17). The isolates of C. albicans used were recovered from the blood of patients with systemic candidosis (CA30 and CA34), or from the oral cavity of an infant with thrush (AK785). Unless otherwise noted, Candida cells were grown aerobically (without shaking) in Sabouraud dextrose broth (SDB; Difco Laboratories, Detroit, MI) for 18 h at 37°C.

Continuous-flow (CF) cultures. The CF cultures were maintained in an anaerobic chamber essentially as described by Freter et al. (9,10). The growth medium, flow rate, inoculation, and operation of the CF culture is described in detail in a previous paper (17). To study the passage of C. albicans through CF cultures, Candida cells were inoculated and viable counts were made by plating samples of serial 10-fold dilutions onto Sabouraud dextrose agar (SDA; Difco) supplemented with chloramphenicol (100 μg ml^{-1}) and streptomycin (100 μg ml^{-1}). The plates were incubated at 37°C and observed for up to 5 days to determine the number of viable Candida. The effluent from CF cultures of intestinal microflora for use in subsequent experiments were collected in a flask kept at ≤4°C, to prevent changes due to bacterial growth occurring after the material had left the growth tube (10). Filtrates from this material were prepared by filtration through 0.22 μm Millipore filters inside the anaerobe chamber. In other experiments, filtrates were prepared from CF culture effluent, or from culture fluid removed directly from the tube as described previously (17).

Growth studies. Growth curves of C. albicans in filtrates of the effluent from CF cultures and in sterile CF culture broth medium, were performed under aerobic and anaerobic conditions at 37°C. For some experiments,

nutrients were added to filtrates of CF culture effluent alone or in combination at various pHs, and in other experiments substances were added to sterile CF culture medium to determine what factors might suppress the growth of Candida in the CF culture.

The nutrients added were $(NH_4)_2$ SO_4 (5.0 g l^{-1}), glucose (12.5 g l^{-1}), and biotin (0.001 g l^{-1}). Possible inhibitory factors included the short-chain fatty acids (SCFAs): acetic acid (83.8 mM), propionic acid (24.0 mM), isobutyric acid (4.7 mM), butyric acid (25.0 mM), isovaleric acid (8.9 mM), and valeric acid (1.8 mM), neutralized to pH 6.8 with NaOH. Five-ml cultures were incubated aerobically and anaerobically at 37°C in 18 x 150 mm tubes and stirred continuously with the same magnetic stirring fleas used in the CF culture apparatus. Growth was assessed using an LC-55 spectrophotometer (Perkin-Elmer, Coleman Instrument Division, Maywood, Ill.) at 600 nm. In some instances, samples of the material were spread onto SDA to determine if Candida remained viable in tubes that showed no visible growth.

Inhibitory activity of short-chain fatty acids (SCFA). Each SCFA (acetic, propionic, isobutyric, butyric, isovaleric and valeric acids) was prepared in CF culture medium at various concentrations starting at double its CF concentration. The pH of the broth was adjusted to 6.8 with NaOH, and each broth was filter

sterilized through 0.22 μ Millipore filters. Serial 2-fold dilutions in CF broth were made, 5-ml broths were then inoculated with 0.1 ml of a C. albicans suspension to give a final concentration of 10^5 cells ml^{-1}, and incubated aerobically at 37°C for up to 5 days to determine the minimal inhibitory concentration (MIC). The cell suspension was prepared by diluting an 18 h SDB culture of C. albicans to the appropriate concentration in CF broth.

Colonization of conventional and antibiotic-treated mice. To assess the role of mucosal association in long-term implantation of the large intestine, conventional and antibiotic-treated mice were inoculated with C. albicans CA34 and lumen contents and mucosal walls were cultured for C. albicans as described previously (18,19). Animal preparation and inoculation has been described in detail elsewhere (16,18-20). Animals receiving antibiotics were given vancomycin (500 μg ml^{-1}), ampicillin (1 mg ml^{-1}) and gentamicin (100 μg ml^{-1}) (VAG) ad libitum for 5 days in the drinking water prior to Candida inoculation (20).

RESULTS

Survival of Candida albicans in CF cultures. As reported in a previous paper (17), C. albicans is eliminated from the CF culture of mouse cecal flora at a rate indistinguishable to the elimination rate from that of the mouse cecum. By plotting the elimination of C. albicans from various CF cultures against the dilution rate of the system, it was determined whether Candida organisms were being killed, were multiplying, or were simply being "washed-out" with the flow of material through the system. Figure 1a shows the passage of CA30 and CA34 from CF cultures of mouse cecal flora. Candida cells for both isolates were removed/eliminated from CF cultures at rates faster than the dilution rate. When inoculated into CF cultures of human fecal flora, in contrast, these same strains were eliminated at apparently the same flow rate (Figure 1b). Thus in both systems C. albicans failed to implant and was eliminated, but only in CF cultures of mouse cecal flora did "killing" of Candida appear to be occurring. The ability of C. albicans (CA34) to implant and grow in a sterile anaerobic CF culture is shown in Figure 2. In this instance, C. albicans was initially removed (for 20 h) from the system with the flow rate, but thereafter Candida cells began to multiply and maintained a steady population of 10^4 cells ml^{-1}. This steady state condition was maintained for longer than 2

weeks.

It is well known that C. albicans cannot easily be implanted into an established intestinal microflora, but that Candida populations can be maintained if given prior to or during the succession and development of the flora (eg, in infant mice). To study this phenomenon, C. albicans CA34 was inoculated into sterile CF cultures simultaneously with 1.0 ml of an established CF culture of mouse cecal flora that had been shown to rapidly eliminate C. albicans CA34. In this experiment, C. albicans was removed from the system with the flow rate for about the first 24 h, but thereafter was removed at a much slower rate (Figure 2). This indicates that C. albicans had become implanted and was multiplying (albeit slowly) in the CF culture in the presence of a functional indigenous microflora. It should be noted, however, that the Candida population was eventually eliminated as the microflora reached equilibrium. To confirm that the bacterial flora was functioning properly, a sample was drawn from the CF culture to determine whether characteristic SCFAs were being produced. It was found that the predominant acids were present, however, both the total and individual levels of SCFAs were present in lower quantities than that found in the donor CF culture. This was not surprising since the microflora had not yet reached equilibrium. Furthermore, phase-contrast microscopy revealed that the predominant bacterial flora was indeed maintained.

Inhibitory mechanisms. To study the mechanisms that were responsible for causing the inhibition of C. albicans in CF cultures of conventional cecal microflora or human fecal microflora, the effluent from these cultures were sterilized by filtration, inoculated with C. albicans (final concentration 10^5 cells ml^{-1}), incubated as static cultures aerobically and anaerobically at 37°C. The growth was monitered for up to 5 days compared to that in sterile CF culture medium. As is shown in Figures 3 a&b, C. albicans was unable to multiply in the culture filtrates from CF cultures of mouse or human intestinal microflora. This was true whether the cultures were incubated aerobically or anaerobically. To determine if the inability of C. albicans to multiply was simply due to the depletion of nutrients, carbon and nitrogen sources, vitamins and trace elements were added to the effluent (Figures 3 a&b). It was found that the inability of Candida to multiply in CF culture filtrates could not be reversed by adding any of these nutrients, either individually or collectively, to the cultures.

Furthermore, this trend was not altered by changing the culture pH (range of 6.0 to 8.0), incubating the cultures aerobically or anaerobically, or both. However, the growth of C. albicans in sterile CF culture medium was reduced when the cultures were incubated anaerobically. Nevertheless, the reduction in growth was not as severe as that in CF culture filtrates. This indicated that

something other than anaerobic condition or substrate depletion, i.e. the production of an inhibitory substance(s), was also involved with controlling the growth of C. albicans in CF cultures.

Figures 3 a&b illustrate the results of experiments in which all six SCFAs (at concentrations resembling those in CF cultures) were added to static cultures of C. albicans. These data indicate that the SCFAs added to fresh medium were able to inhibit the growth of C. albicans, or at least cause a pronounced lag phase to be imparted. By plating samples of these cultures at the end of the experiments, it was determined that Candida organisms remained viable. It was also found that the inhibition of C. albicans was more pronounced when the pH of the medium was lowered to ≤6.0 (data not shown). Furthermore, the inhibitory effect of SCFAs for Candida was found to decrease if the cultures were not stirred. Nevertheless, from the above data it could not be concluded whether one short-chain acid was responsible for the observed suppression, or the mixture acted collectively to inhibit the growth of C. albicans. Therefore, to test this the MIC of each SCFA was determined using the SCFA mixture as a positive control. Table 1 summarizes the results of these experiments. It was found that all of the short-chain fatty acids inhibited the growth of C. albicans, but that some of these did so at levels exceeding those found in the mouse

cecum, human feces, or CF cultures.

Morphology of C. albicans in CF cultures. Candida cells were inoculated into CF cultures in the yeast phase, and were not observed to form germ tubes or "switch" to pseudomycelial or other forms in any of the CF cultures inoculated. This was true whether Candida cells were inoculated alone, simultaneously with intestinal bacteria, or after the intestinal microflora had become established.

Role of adhesion in colonization. As was described above, C. albicans CA34 became implanted (for a short time) in CF cultures of mouse cecal flora only when it was inoculated into the system before or simultaneously with intestinal bacteria. Moreover, it was also noted that while this strain was eliminated from CF cultures of established mouse cecal flora faster than the flow rate (i.e., CA34 was apparently being "killed"). However, when it was inoculated simultaneously with intestinal bacteria C. albicans CA34 was, after an initial period of implantation, removed from the system with the flow rate indicating that it had somehow become "adapted" to the environment. Theoretically, then, C. albicans should be able to remain implanted for longer periods of time if it were able to attach to the wall of the vessel. To test this, the walls of several established CF cultures were cultured after inoculation of C. albicans CA34. Also,

other cultures that were inoculated with C. albicans CA34 simultaneously with intestinal bacteria from an established CF culture were similarly cultured for Candida. Finally, the wall of an established CF culture was cultured after draining its contents, washing it three times and inoculating it with C. albicans CA34. In no instance were viable Candida recovered from the CF culture walls.

Because a glass culture vessel wall is very different from intestinal mucosa, which contain thick layers of mucus that "entrap" Candida (14,20,21), this same type of experiment was repeated using an adult mouse model. For these studies, mice were given a combination of antibiotics (VAG) designed to eliminate most of their intestinal microflora, and subsequently they were challenged with C. albicans. A nonantibiotic-treated control group was also included for comparison. Cecal populations of Candida in the mucosa and lumen contents were then monitered. In the control animals, Candida populations were eliminated rapidly, wheras in the VAG-treated animals C. albicans populations were eliminated at a much slower rate. Moreover, it was observed that in the latter group of animals lumen populations of Candida predominated initially, but as the microflora reestablished mucosal populations predominated.

DISCUSSION

Survival and implantation of C. albicans in the GI tract may be influenced by a number of factors (15), but the presence of an intact indigenous microflora is probably the most important deterrent to whether Candida can colonize the gut (27). Several patient and animal studies support this hypothesis (7,18-21,23-26), and other studies have shown that certain intestinal bacteria are inhibitory to both in vitro growth and GI colonization and dissemination by C. albicans (7,17-21,28,29). However, while the importance of the intestinal microflora has been noted for a number of years, the mechanisms which control Candida in the gut have remained somewhat obscure. This is due, for the most part, to the fact that most studies have reported on Candida suppression by a single bacterial species (4,5,11-13,25,29), which cannot be expected to reflect interactions of a complex indigenous microflora (8). The present studies, therefore, examined the mechanisms by which a complex indigenous microflora inhibits the colonization of C. albicans.

The results presented in this paper strongly support the hypothesis that the indigenous intestinal microflora suppresses C. albicans and suggest that the regulation of Candida populations in the GI tract is the result of a complex series of interactions. The present findings also indicate the importance of using a model that simulates,

as close as possible, the ecosystem under study. This may be inferred from the finding that more than one mechanism was found to suppress C. albicans. Thus, studies in which a monoflora of Escherichia coli (or any other single bacterial species) antagonized C. albicans in the gut of gnotobiotic animals (4,5,12,25), or similar studies performed in vitro (12,13,29), should be interpreted cautiously. Nevertheless, the data presented here, together with the findings by earlier workers, indicate further that the indigenous microflora inhibits Candida colonization by two general mechanisms: (i) antagonizing and suppressing the growth of C. albicans and (ii) inhibiting the association of Candida with the intestinal mucosa.

The data concerning the passage of C. albicans through CF cultures, and the growth of Candida in CF culture effluent, show that the intestinal microflora caused C. albicans to remain in a prolonged lag phase (apparently up to several days) and to have a long doubling time. For instance, when C. albicans was inoculated into CF cultures of human fecal flora (Figure 2) yeast cells were removed with the flow rate, whereas in sterile controls Candida cells maintained a steady population of 10^4 cells ml^{-1}. Discussions in the literature of the possible inhibitory mechanisms involved with the suppression of Candida have suggested that anaerobiosis may be important (13,29). However, anaerobic

condition was only partially inhibitory to growth of C. albicans and did not prolong the lag phase as did CF culture filtrates, indicating that other inhibitory mechanisms were also operating to suppress the growth of C. albicans in this system. The inhibitory effect of Candida growth in CF culture filtrates, moreover, could not be reversed by adding carbon or nitrogen souces, vitamins, or trace nutrients (individually or collectively) to CF culture filtrates and incubating the cultures (under varying pHs) aerobically. Thus the suppression of C. albicans in CF cultures was not due solely to depletion of nutrients and anaerobiosis, but appeared to be due to a combination of these factors in addition to the production of inhibitory substances by intestinal bacteria.

To begin to examine other factors which might be involved in the suppression of Candida growth, certain metabolic end-products from the predominant anaerobic microflora were introduced into sterile CF culture medium and the growth of C. albicans was monitored. In a previous publication (17), it was found that both the total and individual levels of short-chain fatty acids present in CF cultures coincided with the quantity of acids found in the large intestine of conventional mice or human feces used to establish the CF cultures. Furthermore, short-chain fatty acids have been shown to inhibit the adhesion of Candida to intestinal mucosa (20).

Because of this, because much has been speculated about the role of short-chain fatty acids in the control of the intestinal microflora (1,2), and since short-chain fatty acids have been shown to be inhibitory to Saccharomyces cerevisiae (2,3), these acids were thought to be likely candidates for inhibitory substances. It was found that short-chain fatty acids were inhibitory to Candida growth. Further comparison between "MICs" for individual acids and "gut levels" or CF culture levels were performed to determine if the entire mixture collectively was necessary for suppression, or whether one or more acids individually could antagonize Candida growth. The results indicate that acetic and butyric acid antagonized Candida growth at levels that are present in the gut and CF cultures. Although it is not known whether the in vitro results of the present study adequately reflect the sensitivity of C. albicans to these acids in the GI tract or CF cultures, it is not likely that inhibitory concentrations of these short-chain fatty acids would be higher. Therefore, these acids are likely to play a role in the suppression of Candida in the gut. A similar argument could be made to suggest that secondary bile acids also play a role in the suppression of C. albicans in the GI tract. For instance, secondary bile acids have been shown to inhibit both the in vitro growth and the adhesion of C. albicans to intestinal mucosa (20).

According to Freter et al. (8,9), however, it is

theoretically impossible to account for the total suppression of a sensitive microorganism (e.g., C. albicans) in the GI tract on the basis of the production of growth inhibitors. If this were the case, constant populations of the sensitive species could only be maintained at precise inhibitor concentration (8). Thus if too much inhibitor were present C. albicans would be eliminated, or if too little were present the Candida population would increase until it became limited by some other mechanism. Although Candida population levels in the GI tract do show some increases or decreases from day to day (7), it may be argued that other inhibitory mechanisms are also involved with the suppression of C. albicans in the gut. Considering that there must be fluctuations in the production of growth inhibitors, and considering that short-chain fatty acids appear only to be fungistatic, this is likely to be the case. It should also be noted that there may be an enormous supply of growth inhibitors that can be produced by the indigenous intestinal microflora, which would be subject to fluctuation. For instance, several breakdown products from the large number of primary dietary or host-derived substrates in the gut may antagonize C. albicans, as might a number of substances synthesized by the microflora itself. Although C. albicans was apparently being "killed" when inoculated into established CF cultures (only mouse cecal flora), it was not "killed" when it was

inoculated before or simultaneously with the microflora. Thus it may be that Candida can adapt itself to these "toxic" substances, which, nevertheless, are probably still inhibitory to Candida growth. This view is consistent with the finding that C. albicans was able to implant itself (albeit only for a short time) in CF cultures of mouse cecal flora.

The reason C. albicans could not permanently colonize CF cultures in large numbers was probably due to a lack of adhesion sites or the inability to attach to the glass vessel in the presence of, for example, short-chain fatty acids (14,15,20). This suggests that adhesion to the gut wall may be an important determinant to colonization for C. albicans, since association with the intestinal mucosa would probably allow Candida to remain in the gut even at a severely depressed growth rate. Moreover, it may also be that colonization of the mucosa would provide a more sheltered site from growth inhibitors. The finding that mucosa-associated populations of Candida predominated after the intestinal microflora reestablished is certainly consistent with this view. Therefore, the ability of the intestinal microflora to inhibit the association of C. albicans with the intestinal mucosa also represents an important defense mechanism that inhibits Candida colonization (and systemic spread from) of the GI tract. The mechanisms by which intestinal bacteria do this have been commented on elsewhere (20).

Our current hypothesis holds that Candida populations are controlled collectively by several factors. First, C. albicans is bombarded by several growth inhibitors (e.g., short-chain fatty acids, secondary bile acids, etc.) that cause Candida cells to remain in a prolonged lag phase and have a long doubling time when entering the GI tract. This alone may cause Candida organisms to be removed from the GI tract due to peristalsis and the flow of mucus; unless, of course, the Candida are able to associate with the mucosa. Second, there are several mechanisms which are operating in concert to inhibit and suppress Candida cells from attaching to the gut wall, including competition for adhesion sites, production of inhibitor substances, prevention of penetration into the mucus gel, and an increased disassociation rate (20). Finally, superimposed upon and modifying this type of regulation is the metabolic competition for limiting nutrients, which probably becomes the predominant control mechanism if Candida cells become implanted in the gut. There is good evidence to suggest that the presence of certain metabolic end-products produced by the predominant anaerobes restricts the range of substrates which a given organism can efficiently utilize for anaerobic growth (9). Thus C. albicans is probably collectively controlled by growth inhibitors and anaerobiosis (which severely depresses its growth rate), competition for mucosal sites, and substrate competition. It is not known to what extent these

mechanisms individually control the growth of Candida in the gut, but the range of factors which can cause and "overgrowth" of Candida in the GI tract suggest that no one single mechanism is responsible for the regulation of C. albicans populations. This may help to explain discrepancies in the literature regarding which organisms of the intestinal microflora are responsible for controlling Candida in the gut.

REFERENCES

1. Argenzio, R.A. 1981. Short-chain fatty acids and the colon. Digest Dis Sci 26:97-99.

2. Bergeim, O. 1940. Toxicity of intestinal volatile fatty acids for yeast and Escherichia coli. J Infect Dis 66:222-234.

3. Bergeim, O., A.H. Hanszen, L. Pincussen, and E. Weiss 1941. Relation of volatile fatty acids and hydrogen sulphide to the intestinal flora. J Infect Dis 69:155-166.

4. Balish, E., and A.W. Phillips. 1966. Growth and virulence of Candida albicans after oral inoculation in the chick with a monoflora of either Escherichia coli or Streptococcus faecalis. J Bacteriol 91: 1744-1749.

5. Balish, E., and A.W. Phillips. 1966. Growth, morphogenesis and virulence of Candida albicans after oral inoculation in the germ-free and conventional chick. J Bacteriol 91:1736-1743.

6. Clark, J.D. 1971. Influence of antibiotics or certain intestinal bacteria on orally administered Candida albicans in germ-free and conventional mice. Infect Immun 4:731-737.

7. Ekenna, O., and R.J. Sherertz. 1987. Factors affecting colonization and dissemination of Candida albicans from the gastrointestinal tract of mice. Infect Immun 55:1558-1563.

8. Freter, R. 1983. Mechanisms that control the microflora in the large intestine. In: D.J. Hentges (Ed.), Human intestinal microflora in health and disease, p. 33-54. Academic Press, Inc., New York.

9. Freter, R., H. Brickner, M. Botney, D. Cleven, and A. Aranki. 1983. Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora. Infect Immun 39:676-685.

10. Freter, R., E. Stauffer, D. Cleven, L.V. Holdeman, and W.E.C. Moore. 1983. Continuous-flow cultures as in

vitro models of the ecology of large intestinal flora. Infect Immun 39:666-675.

11. Hummel, R.P., M.P. Maley, P.W. Miskell, and W.A. Altemeier. 1975. Suppression of Candida albicans by Escherichia coli. J Trauma 15:413-418.

12. Hummel, R.P., E.J. Oestreicher, M.P. Meley and B.G. MacMillan. 1973. Inhibition of Candida albicans by Escherchia coli in vitro and in the germfree mouse. Journal of Surgical Research 15: 53-58.

13. Isenberg, H.D., M.A. Pisano, S.L. Carito, and J.I. Berkman. 1960. Factors leading to overt monilial disease. I. Preliminary studies of the ecological relationship between Candida albicans and intestinal bacteria. Antibiot Chemo 10:353-363.

14. Kennedy, M.J. 1988. Adhesion and association mechanisms of Candida albicans. In M.R. McGinnis (ed): Current Topics in Medical Mycology, Vol 2. New York, Springer-Verlag, pp 73-169.

15. Kennedy, M.J. 1987. Role of motility, chemotaxis and adhesion in microbial ecology. Annals of New York Acad Science 506:260-273, 1987.

16. Kennedy, M.J., P.S. Bajwa, and P.A. Volz. 1982. Gastrointestinal inoculation of Sporothrix schenckii in mice. Mycopathologia 78:141-143.

17. Kennedy, M.J., A.L. Rogers, and R.J. Yancey. 1988. An anaerobic continuous-flow culture model of interactions between intestinal microflora and Candida albicans. Mycopathologia 103:125-134.

18. Kennedy, M.J., and P.A. Volz. 1983. Dissemination of yeasts after gastrointestinal inoculation in antibiotic-treated mice. Sabouraudia 21:27-33.

19. Kennedy, M.J., and P.A. Volz. 1985. Effect of various antibiotics on gastrointestinal colonization and dissemination by Candida albicans. Sabouraudia: J Med Vet Mycol 23:265-273.

20. Kennedy, M.J., and P.A. Volz. 1985. Ecology of Candida albicans gut colonization: Inhibition of Candida adhesion, colonization, and dissemination from the gastrointestinal tract by bacterial antagonism. Infect Immun 49:654-663.

21. Kennedy, M.J., P.A. Volz, C.A. Edwards, and R.J. Yancey. 1987. Mechanisms of association of Candida albicans with intestinal mucosa. J Med Microbiol 24:333-

341.

22. Krause, W., H. Matheis, and K. Wulf. 1969. Fungaemia and funguria after oral administration of Candida albicans. Lancet, 1, 598-599.

23. Miles, M.R., L. Olsen, A.L. Rogers. 1977. Recurrent vaginal candidiasis: Importance of an intestinal reservoir. J Am Med Assoc 238:1836-1837.

24. Myerowitz, R.L., G.J. Pazin, and C.M. Allen. 1977. Disseminated Candidiasis: Changes in incidence, underlying diseases, and pathology. Am J Clin Path 68:29-38.

25. Nishikawa, T., H. Hatano, N. Ohnishi, S. Susaki, S. and T. Omura. 1969. Establishment of Candida albicans in the alimentary tract of the germfree mice and antagonism with Escherichia coli after oral inoculation. Jap J Microbiol 13:263-276.

26. Nystatin Multicenter Study Group. 1986. Therapy of candidal vaginitis: The effect of eliminating intestinal Candida. Am J Obstet Gynecol 155:651-655.

27. Odds, F.C. 1988. Candida and Candidosis, University Park Press, Baltimore.

28. Ozawa, K., K. Jabu-Uchi, K. Yamanaka, Y. Jamachita, K. Ueba, and T. Micwatani. 1979. Antagonistic effects of Bacillus nato and Streptococcus facealis on growth of Candida albicans. Microbiol Immunol 23:1147-1156.

29. Paine, T.F., Jr. 1958. The inhibitory activity of bacteria on Candida growth. Antibiot Chemother 8:273-281.

30. Stone, H.H. 1974. Studies in the pathogenesis, diagnosis and treatment of Candida sepsis in children. J Pediatr Surg 9:127-133.

31. Stone H.H., C.E. Geheber, L.D. Kolb, and W.R. Kitchens. 1973. Alimentary tract colonization by Candida albicans. J Surg Res 14:273-276.

32. Stone, H.H., L.D. Kolb, C.H. Currie, C.E. Gebeher, and J.F. Cuzzell. 1974. Candida sepsis: pathogenesis and principles of treatment. Ann Surg 179:697-711.

Table 1. Minimal inhibitory concentrations for short-chain fatty acids against Candida albicans.

Strain identification	MIC (mM)					
	Acetic	Propionic	Isobutryic	Butyric	Isovaleric	Valeric
CA30 SDB grown	40	40	20	10	20	10
CAD30 LBC yeast grown	40	40	20	20	10	5
CA30 "singlets"	40	40	>20	40	20	10
CA34 SDB grown	40	20	20	10	10	2.5
"Gut levels" (mM)	116.1	20.4	4.5	20.8	3.8	1.3

Figure 1 a&b. Passage of C. albicans through continuous-flow cultures of mouse (a) and human (b) intestinal flora.

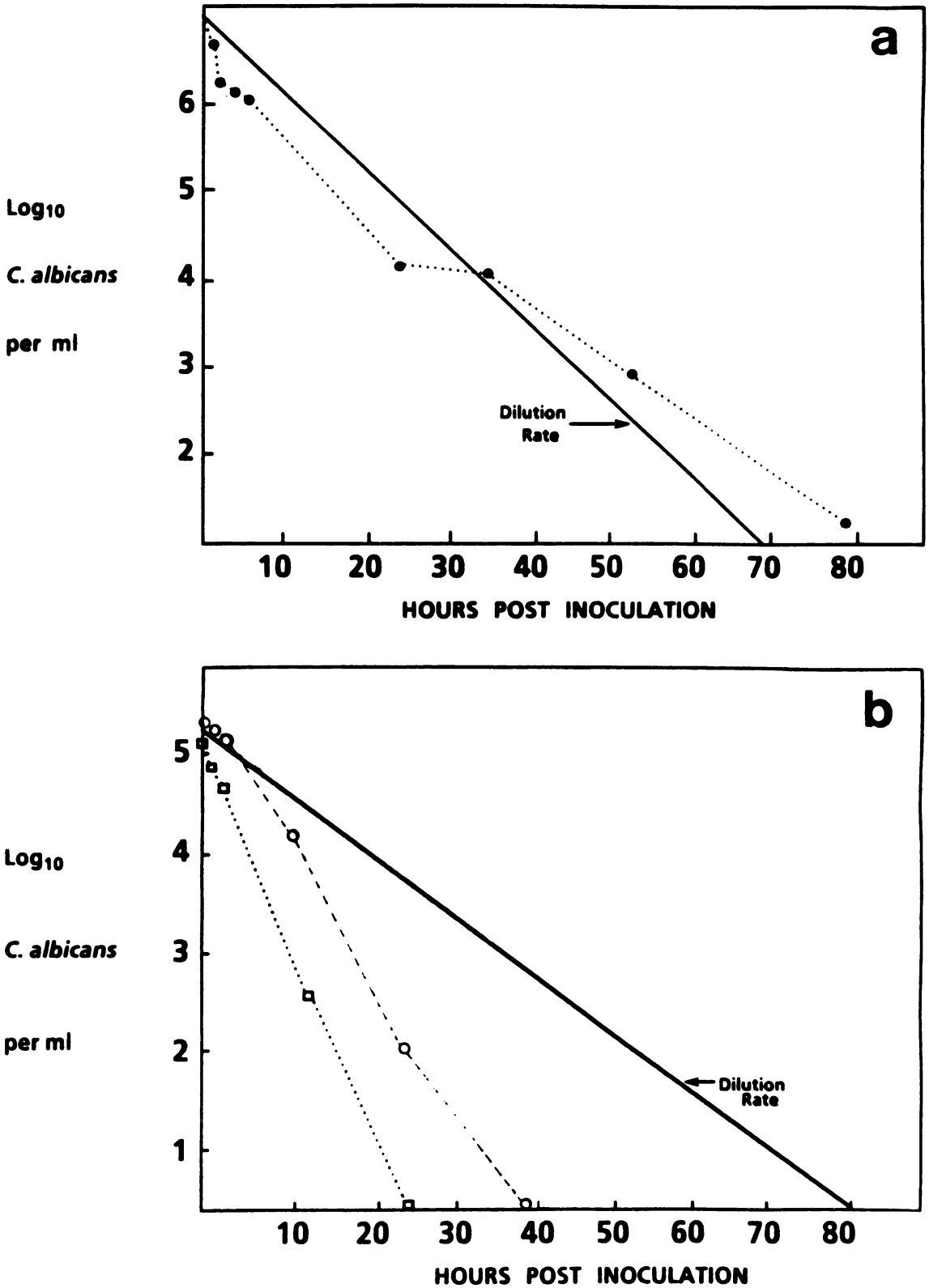


Figure 2. Survival and implantation of C. albicans through continuous-flow cultures of established mouse cecal bacteria (□), freshly inoculated cultures (△), or sterile anaerobic control cultures (○).

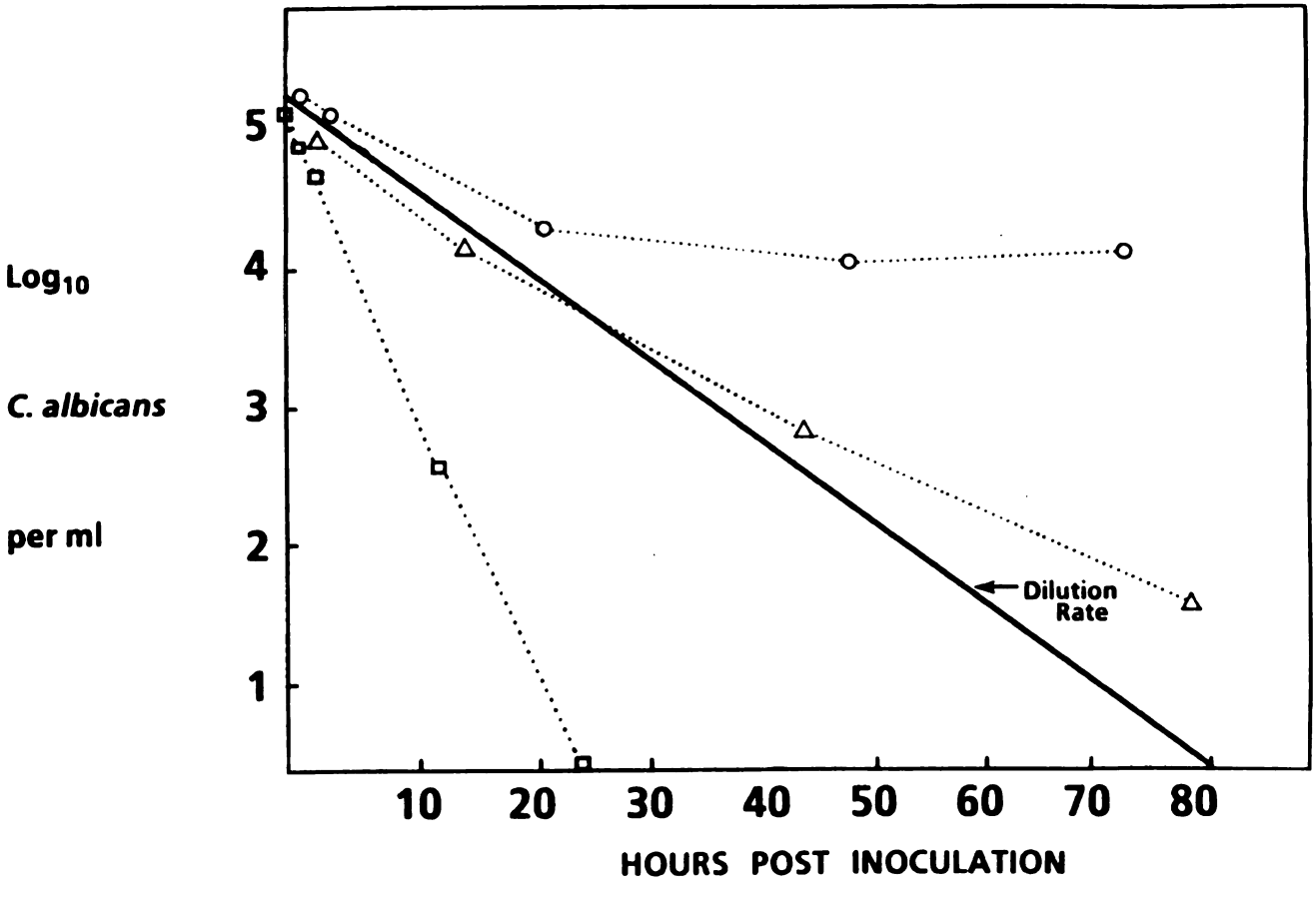
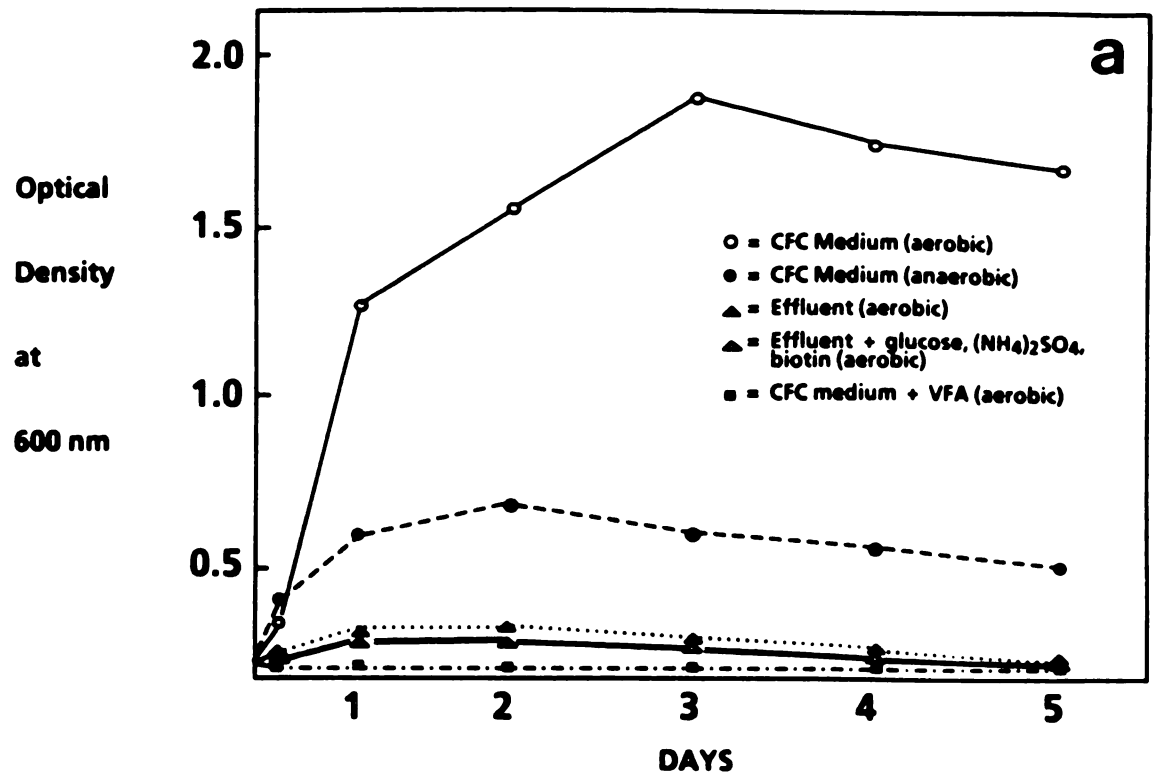
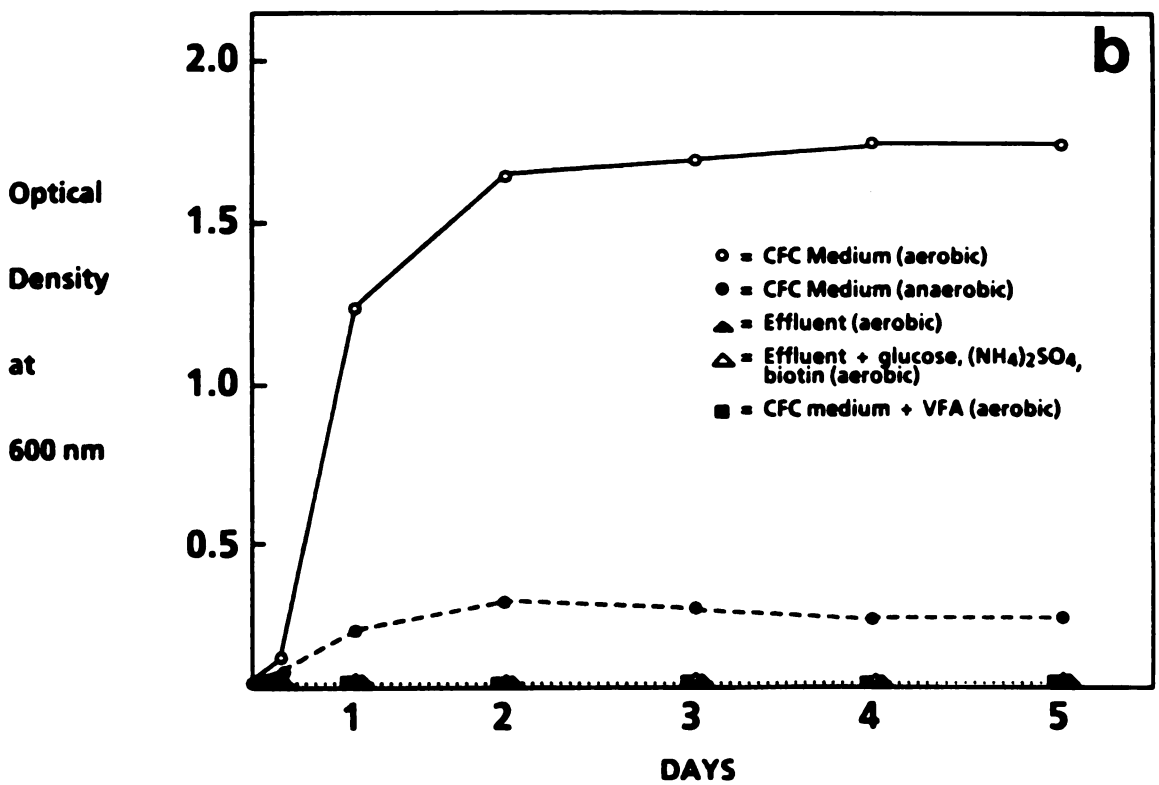


Figure 3 a&b. Growth of Candida albicans in filtrates of continuous-flow cultures of mouse cecal bacteria (a) and human fecal flora (b).





DISCUSSION

DISCUSSION

ADHESION AND ASSOCIATION MECHANISMS OF CANDIDA ALBICANS

The "attachment" of C. albicans to both biological and nonbiological surfaces is considered an important event in colonization and pathogenesis of humans (Kennedy, 1987 & 1988). Moreover, adhesion and aggregation may also be an important virulence factor that allows this fungus to resist host defenses and antifungal therapy. Because of the obvious importance of Candida adhesion and aggregation to human health, then, numerous in vitro and in vivo models have been developed to study the adhesion of C. albicans to various nonbiological surfaces and host tissues (Kennedy, 1988). Several models, for instance, have been developed to study the adhesion and association of C. albicans to mucosal surfaces, certain organs, and prosthetic devices. These models have been used with some success to characterize C. albicans adhesion and association mechanisms, and the role attachment plays in colonization and pathogenesis of this important pathogen (Kennedy, 1988). However, the use of many of these same models has also led to several discrepancies in the literature as to the cell wall component(s) of C. albicans that mediates its attachment to, for example, epithelial cells as well as to the mechanism(s) of invasion of mucosal and endothelial surfaces (Kennedy, 1988). It has

been suggested, for instance, that a mannose containing moiety, probably a mannoprotein (Critchley & Douglas, 1987; McCourtie & Douglas, 1985a), is the cell wall adhesin of C. albicans (Lee & King, 1983; Sandin, 1987 a&b; Sandin & Rogers, 1982; Sandin et al., 1982), whereas analogous studies have suggested that chitin is the adhesin (Segal et al., 1982). Other studies suggest that there may be more than one adhesin (Kennedy et al., 1987; McCourtie & Douglas, 1985a; Sandin, 1987a) and at least one report suggests that the adhesion of C. albicans may not be mediated by a specific adhesin-receptor interaction but may be entirely nonspecific (Reinhart et al., 1985).

The obvious means of resolving such extreme positions lies first in the development of models that closely mimic the infection or adhesive process as it occurs in the natural host or environment and second in the proper critical interpretations and caution that must be applied to the data obtained with adhesion models. As has been described in detail elsewhere (Kennedy, 1988), there are numerous methodologic pitfalls that accompany the use of adhesion models. A survey of the literature on the adhesion of C. albicans to epithelial cells showed that this fact has not generally been appreciated (Kennedy, 1988). The reasons for this lie not only in the fact that few critical studies have examined experimental factors that influence adhesion data but also because adhesion models to study fungal attachment mechanisms have been in

use for a relatively short period of time. That is to say, knowledge of fungal adhesion and aggregation mechanisms and the factors that influence fungal attachment is still in an early state. Thus at present, one can still only speculate at the actual contribution of many of the numerous attachment mechanisms that have been identified using various adhesion models.

Although any adhesive reaction observed for C. albicans in an adhesion model must be viewed as a real event, rigorous proof that adhesion is necessary for colonization, pathogenesis, or both for a number of adhesion mechanisms that have been identified to date must still be proven. For instance, if an adhesion mechanism is observed in an experimental animal model, it cannot apriori be assumed to be important in human disease (Kennedy, 1988). Likewise, any adhesive reaction observed for C. albicans in a simple adhesion assay performed in vitro should not automatically be assumed to represent adhesion in humans. This is true whether the adhesion is in the oral cavity, or to the surface of a prosthetic device. Moreover, at present there seems to be little appreciation for the complexities of host-fungal interactions including the interdependence of factors that regulate colonization, the various factors that can regulate adhesion and aggregation, and the redundancy of some of these mechanisms. For example, as will be discussed below, there is more than one mechanism by which

C. albicans can interact with a surface so as to reduce its rate of removal. Although numerous other examples of the complexities of host-fungal interactions could be given, all of these point to the fact that a satisfactory understanding of the role any one mechanism of adhesion has in colonization or pathogenesis comes from: (i) an elucidation of the biochemical and molecular basis of that mechanism and (ii) an understanding of its precise interaction with all the other mechanisms that collectively regulate the sequential processes of colonization and pathogenesis. Consequently, it is suggested that the study of fungal attachment should include: (i) the development of model systems that closely mimic the physicochemical environment of the host and (ii) models that address the interaction of adhesion mechanisms with other factors that are equally, and in some instances more, important in colonization and pathogenesis.

As noted above, efforts to study C. albicans adhesion and association mechanisms have led to the development of numerous models. These models have included both in vitro and in vivo models, and have included models to study interactions between C. albicans and host tissues or nonbiological surfaces as well as intrageneric and intergeneric microbial aggregate formation. In addition, several models have been developed to study various cell surface properties of both yeast and hyphal forms of C. albicans that may play a role in adhesion and aggregation.

Although the use of adhesion models, and simple *in vitro* assays in particular, has led to several erroneous conclusions regarding the nature of how *C. albicans* "attaches" to host tissues or the role certain adhesive reactions play in colonization and pathogenesis, there can be no reasonable doubt that very little progress in the understanding of *Candida* attachment could be made without their use (Kennedy, 1988).

Nevertheless, it is important to note that there are also a number of limitations that accompany the use of adhesion models. For instance, the pathogenesis of any fungal infection, whether it involves *C. albicans* or some other fungal pathogen, is the result of a complex series of separate but interdependent interactions that cannot all be reproduced *in vitro*. Consequently, simple *in vitro* assays may yield little information to the more important question of the role of *Candida* attachment in colonization and pathogenesis. Moreover, adhesion may only represent one step in the sequential process of colonization and in this process one type of adhesion (i.e. initial or reversible adhesion) may yield itself to yet another type of adhesion (i.e., secondary or permanent adhesion). Thus in some instances adhesion may only represent a transient step in colonization or pathogenesis. Furthermore, in the host, *C. albicans* may attach to surfaces by more than one attachment mechanism. As one example, there appears to be at least five distinct mechanisms by which *C. albicans* can

associate with intestinal mucosa (Kennedy et al., 1987).

The complexity of Candida-mucosal association, therefore, makes it difficult to devise a single model system (e.g., adhesion to isolated intestinal epithelial cells) which can be relied on to duplicate in vitro the process as it occurs in vitro (Kennedy, 1988). Therefore, while in vitro systems remain valid tools to identify, differentiate and study various adhesion and aggregation mechanisms of Candida at the molecular level, proper critical interpretation as well as caution must be applied to the data when extrapolating to the situation found in vivo or in the natural environment. It may be, then, that the continued use of any in vitro model of adhesion may require demonstration of the similarity of the adhesion or aggregation mechanism(s) in vitro to the process as it occurs in humans (Kennedy, 1988).

A complete understanding of the physicochemical or molecular basis of C. albicans adhesion or aggregation, and the role that a particular mechanism plays in colonization or pathogenesis, may only be gained by studying each aspect of adhesion (e.g., the mechanism(s) of adhesion versus the role of attachment in colonization and pathogenesis) separately in different adhesion models. For instance, it may be necessary to initially study the adhesion of C. albicans to intestinal mucus gel and epithelium seperately in vitro, but thereafter to proceed to more complex in vitro models (e.g., intestinal slices),

followed finally by studies in experimental animals. Only by following this type of strategy can one be sure of the relevence of the adhesive reaction studied *in vitro*. Furthermore, only by using several models to study adhesion and the role adhesion plays in colonization and pathogenesis can important information regarding the regulation and synthesis of adhesins as well as factors that might influence (either positively or negatively) adhesion be gained. An examination of factors present in the gut of conventional mice but absent in the gut of antibiotic-treated mice, for instance, has revealed that several bacterially-derived substances (produced by the metabolic activity of the indigenous microflora) may inhibit the attachment of *C. albicans* to intestinal epithelium in the noncompromised host (Kennedy & Volz, 1985a). Thus factors present in a given ecosystem may be as important as the presence of an adhesin itself in controlling adhesion. Finally, this type of strategy has previously been used to show that not all attachment mechanisms observed *in vitro* models of adhesion (Freter & Jones, 1976; Jones *et al.*, 1976; Jone & Freter, 1976) are involved with the association of microorganisms with host tissue *in vivo* (Freter *et al.*, 1981 a&b).

There are a number of experimental factors that can influence the adhesion or aggregation of *C. albicans in vitro* and *in vivo* (Tables 6 & 7). Although a number of factors have been shown in the present studies to

Table 6. Factors affecting fungal aggregation and adhesion to biological and nonbiological surfaces *in vitro**

Factor	Examples
Environmental factors	Agitation speed Antibiotics Assay medium Assay temperature Incubation time pH of assay medium Ionic strength Yeast cell concentration
Epithelial factors	Body site of origin Cell type Donor Indigenous microflora Viability
Fungal factors	"Cell-type" or "phenotype" Coadhesion Morphology Strain Viability
Growth factors	Growth phase Medium composition Medium viscosity Temperature

*Modified from Kennedy (1988).

Table 7. Factors influencing fungal aggregation and adhesion *in vivo**

Factor	Examples
Host factors	Antimicrobic therapy Body site Diet Environmental factors (eg, pH) Hormonal status Immunologic status Number and type of receptors available Physiologic status
Indigenous microflora	Alteration of physiochemical nature of environment Colonization of adhesion sites Modification of substrate Production of inhibitors
Fungal factors	"Cell-type" or "phenotype" Concentration and type of adhesins synthesized Enzyme production (eg, proteinase(s) Germination Morphology Surface properties

*Modified from Kennedy (1988).

influence the adhesion of C. albicans to BECs and plastic (Kennedy, 1988), growth conditions are probably the most important (Kennedy, 1988; McCourtie & Douglas, 1981 & 1984). As can be noted from Table 6, not only can the phase of growth modify adhesion but so can the growth temperature and growth medium composition. Of the factors that can influence Candida adhesion and aggregation, growth medium has been suggested to be the most important (Kennedy, 1988). While this may seem intuitively obvious, it is somewhat surprising to note that over 15 different media have been used in the literature for studies on C. albicans adhesion (Kennedy, 1988). McCourtie and Douglas (1981 & 1984) noted in studies in the early 1980's that growth of C. albicans in media that enhanced adhesion to BECs and acrylic correlated with changes in yeast cell surface composition. Yet a recent survey of the published data on the adheion of C. albicans to BECs showed that although these findings are often cited they have not generally been appreciated (Kennedy, 1988). Of particular interest is the fact that very few investigators have used the same growth medium, and that there are major differences between media used by those investigators proposing that different cell surface moieties are responsible for C. albicans adhesion (Kennedy, 1988). Because of this, because there was no way of comparing previously published adhesion data directly, and since there has been little or no agreement as to the selection

of the assay conditions for a standardized buccal epithelial cell adhesion assay for Candida, the effects several growth media (as well as various growth conditions) had on C. albicans adhesion to BECs and plastic were studied.

To determine the effect growth medium had on the adhesion of Candida to BECs, a virulent and adhesive strain of C. albicans (AK785) isolated from the mouth of a 1 week-old female with oral thrush was grown in several media and examined for its adhesiveness to BECs. The ability of C. albicans to adhere to BECs was found to be highly dependent on the growth medium used for cell growth. As can be noted from the Table 2 from article 1, Candida cells grown in undefined liquid media at 37^{o}C were usually less adhesive than those cells grown in defined media at the same temperature. However, for one defined medium (YNB) the production of highly adhesive cells was dependent upon the type of carbohydrate present in the medium during cell growth. This finding confirms the earlier results that cells of C. albicans grown in YNB + 500 mM galactose are significantly more adhesive than cells grown in YNB + 50 mM glucose (McCourtie & Douglas, 1981 & 1984). Moreover, significant differences in adhesion of C. albicans to BECs were noted when the cells were grown in the same complex medium from different manufactures as well as from different lots of medium from the same manufacture. Other differences in cell growth

parameters, such as growth temperature and whether the cells were grown on agar or in broth, also had a significant effect on C. albicans adhesion data. All of these factors also modified Candida cell surface hydrophobicity and the ability of C. albicans to attach to BECs indirectly by yeast-to-yeast coadhesion.

To determine whether differences in morphology, cell wall ultrastructure or various cell surface features could account for some of the differences in C. albicans adhesion and coadhesion noted, Candida cells were also examined by SEM and TEM. Although several morphologic characteristics were noted, only the presence of tiny germtube-like structures, which were almost imperceptible by light microscopy, and very small blastospores appeared to correlate with increased yeast adhesiveness. Of all the cell types observed in that study, small blastospores were the most adhesive followed by yeast cells with short germtube-like structures, yeast cells with longer germtubes, and pseudomycelia and yeast cells, respectively. Furthermore, although differences were noted in cell wall thickness, the number of distinct cell wall layers, and the presence of an extra electron dense cell wall layer, none of these differences appeared to account for either an increase or decrease in adhesion, coadhesion or cell surface hydrophobicity. Similarly, the presence of an outer cell wall floccular material, which previously was shown to confer an increase in C. albicans

adhesion to BECs (McCourtie & Douglas, 1984), did not appear to correlate with Candida adhesiveness or cell surface hydrophobicity.

These findings clearly demononstrate the importance of growth parameters on the morphology, budding pattern, cell wall ultrastructure and various surface properties of C. albicans. These include not only cell surface hydrophobicity but also cell surface topography and the ability of Candida cells to attach to BECs either directly (adhesion) or indirectly (coadhesion). Although these data suggest that growth conditions can significantly influence C. albicans adhesion data, they do not allow definitive conclusions to be drawn regarding the increased adhesiveness of small blastoconidia or cells with germtubes. Nevertheless, there are at least three possible mechanisms which could account for this. (i) It is apparent that there are major changes in the organization of the cell wall at the site of germtube formation (Cassone et al., 1973; Schertwitz et al., 1978; Tronchin et al., 1984), which has been shown to occur in infected tissue (Montes & Wilborn, 1968 & 1985). These changes may allow for a concentration of adhesins or the production of different adhesins, both of which may increase the adhesiveness of C. albicans (Kennedy, 1988). There is some evidence that C. albicans does produce more than one adhesin (Kennedy et al., 1987; McCourtie & Douglas, 1985a; Sandin, 1987b). Furthermore, recent

studies have shown that germtubes of C. albicans possess cell surface antigens which are distinct from those found on the surface of budding yeast cells (Brawner & Cutler, 1985 & 1986; Ponton & Jones, 1986; Smail & Jones, 1984; Sundston & Kenny, 1984; Sundstrom et al., 1987). It should be noted, however, that it is not known whether these antigens serve as adhesins or not, but these findings again emphasize that there are major differences between cell wall composition of yeast cells and germtubes (Cassone et al., 1973). This fact becomes very important, then, when selecting a growth medium for C. albicans adhesion studies. (ii) There is evidence to suggest that production of certain enzymes (e.g., proteinases) in concert with germtube formation may facilitate adhesion (Ghannoum & Elteen, 1986). (iii) It is likely that the germtubes, which have very small radii of curvature compared to the yeast cell, would favor adhesion by reducing repulsive interactions that occur during adhesion between cells (Kennedy, 1988). The latter factor may also help to explain why very small blastospores of C. albicans were found to be significantly more adhesive to BECs than were "normal" size yeast cells that did not possess germtubes.

The differences noted in C. albicans cell wall ultrastructure and adhesion to BECs with respect to various growth parameters, especially growth medium, raise questions regarding the location and biochemical nature of

the Candida adhesin(s). For instance, at least two morphologic classes of Candida adhesins (floccular and fibrillar [reviewed in Kennedy, 1988]) have been noted in the literature, both of which have been observed to mediate C. albicans adhesion in vivo (Barnes et al., 1983; Calderone et al., 1984; Montes & Wilborn, 1968 & 1985). In contrast, it has also been shown that the adhesion of C. albicans to epithelial cells was mediated by cells that did not contain such outer surface layers (Calderone et al., 1984). Although cells of C. albicans with a floccular or fibrillar cell wall layer have been observed to apparently mediate adhesion to various tissues, as have Candida cells with no apparent adhesive appendages, no study to date has determined the similarity of adhesins produced in vitro and in vivo. These will be key future experiments for the selection of growth conditions and assay parameters for the design and standardization of relevent and useful adhesion models. This seems especially true in view of the finding that the presence of either floccular or fibrillar structures on the cell surface of C. albicans does not, by itself, indicate that these structures play a role in either adhesion.

Candida cells grown in TSB, for instance, were found to contain an outer floccular cell wall layer, and were relatively nonadhesive to BECs. In contrast, growth of C. albicans in YNB + gal produced cells that contained an outer floccular layer which were highly adhesive to BECs.

Furthermore, growth of C. albicans in other media produced cells that were highly adhesive to BECs that had relatively thin cell walls with no "extra" outer layers. Thus, these findings might be interpreted to suggest that neither of the aforementioned structures is required for adhesion of C. albicans to BECs, although they may mediate attachment. It may also be that after the initial binding, Candida cells modify their cell walls, and floccular or fibrillar structures are synthesized which stabilize and strengthen adhesion (Kennedy, 1988). This is supported by the data of Tronchin et al. (1984) who noted the reorganization and proliferation of an external cell wall layer of C. albicans during adhesion to BECs. The observation that the floccular layer was produced only amorphously around the cell in some instances, and that not all cells in a population had the material, however, may also correlate with the suggestion that adhesins are distributed unequally in populations of C. albicans (McCourtie & Douglas, 1985b). This suggests that the biochemical nature of the "floccular" layer noted between cells grown in various media may be very different, and some but not all such structures may contain Candida adhesins (Kennedy, 1988).

The findings that growth of C. albicans in various media produced significant differences in cell wall ultrastructure, and presumably cell wall composition, may also clear up previous contradictory results in the

literature regarding the nature of the cell wall component that serves as the Candida adhesin (Kennedy, 1988). For instance, Segal and co-workers (Lehrer et al., 1983; Segal et al., 1982) have suggested that cell wall chitin, which is located at the innermost portion of the cell wall or at bud scars (Poulain et al., 1985; Tronchin et al., 1981), may serve as the adhesive component. In contrast, Douglas and co-workers have suggested that mannoprotein is the Candida adhesin (Critchley & Douglas, 1987; McCourtie & Douglas, 1985). It is important to note that the growth media used by these two groups is extremely different and yields cells that have a significantly different cell wall ultrastructure. Growth of C. albicans in the former medium (yeast extract medium) yielded cells of C. albicans that possessed the thinnest cell wall with the fewest apparent layers, whereas the latter medium (yeast nitrogen base supplemented with 500 mM galactose) yielded cells with thicker cell walls that possessed an outer floccular layer. From these data, and the data presented above, it is suggested that at least three different adhesins exist on the surface of C. albicans that mediate its attachment to BECs: (i) a cell wall adhesin (demonstrated best when cells are grown in LBC broth), (ii) an adhesin in the floccular layer (YNB + 500 mM galactose), and (iii) an adhesin expressed below the surface that becomes evident when the outer electron layer is thinned (yeast extract medium). The variations in cell wall ultrastructure and

adhesion noted from these studies, then, underscore the capacity of this organism for cell surface modification *in vitro* and the importance of standardizing fungal adhesion assays. Moreover, one is left to wonder which, if any, of the numerous studies reported in the literature mimic adhesion to buccal epithelial cells as it occurs *in vivo*.

Another important factor that may significantly alter fungal adhesion and aggregation data comes from the finding that *C. albicans* is capable of switching, reversibly and at a high frequency between several phenotypes that are readily distinguishable by the size, shape, and color of colonies formed on agar at 25°C (Slutzsky *et al.*, 1985 & 1987). The differences noted in colony characteristics apparently were due to the dramatic difference in cell size, shape, and budding pattern (Slutzsky *et al.*, 1985 & 1987). Two of these phenotypes ("white" and "opaque") were examined for their ability to attach to BECs and plastic, and to compare their cell surface hydrophobicities. "White cells" are round to ellipsoidal and exhibit a budding pattern similar to most strains of *C. albicans*, whereas "opaque cells" are elongate, or bean shaped, and exhibit a different budding pattern. As was shown in the studies described above, white cells were found to be significantly more adhesive to BECs than were opaque cells. In contrast, opaque cells were found to be twice as hydrophobic as white cells as was the percentage of opaque cells that attached to BECs

by coadhesion. The differences in adhesion to plastic between the two phenotypes were not statistically significant and there was no distinct trend to suggest which phenotype might be more adhesive to plastic. These results indicate that several factors are involved in the adhesion of C. albicans to plastic, and confirm the hypothesis that cell surface hydrophobicity is of minor importance in direct adhesion to epithelial cells but that it may contribute to indirect attachment to epithelial cells by promoting yeast coadhesion. Moreover, these findings revealed that under identical growth conditions, cell surface topography, cell surface hydrophobicity, and adhesion of C. albicans was significantly altered depending on the phenotypic state of the organism tested.

The above findings further stress the importance of culture conditions in the study of fungal adhesion and aggregation as well as maintaining a proper perspective on the data obtained from simple in vitro adhesion assays. For instance, while the opaque phenotype of C. albicans may predominate in some environment (e.g., soil or water) other than the host, differences in the adhesive and cell surface properties between white and opaque phenotypes may yield important information about how Candida cells attach to biological and nonbiological surfaces. Therefore, although the importance of the white-opaque transition in colonization and infection has not been established, the white-opaque system lends itself as a useful tool to aid

in the characterization of C. albicans adhesion mechanisms.

In view of the findings described above, and the descrepancies regarding which surface component(s) serves as the Candida adhesin(s), the need for a defined and reproducible assay to study adhesion of C. albicans to BECs is very apparent. The fact that over 15 different growth media have been used to cultivate Candida cells for adhesion studies, coupled with the numerous other variations in experimental factors used by different investigators, further stresses this point but also suggests that at present there is little, if any, agreement on the selection of criteria required for the standardization of a buccal cell adhesion assay (Kennedy, 1988). Because such an assay is important to describe C. albicans adhesion at the molecular level and to confirm the role an adhesion mechanisms might play in colonization or pathogenesis, the beginnings of a standardized assay are outlined in Table 8 and the paragraphs below.

The most important factor to be considered, and the only one that will be discussed here, is the selection of a culture medium and growth conditions (Kennedy, 1988). It is clear from the data discussed above, that uniformity of adhesion results between lots of complex commercial media (e.g., Sabouraud dextrose broth, which apparently has been the medium of choice [see Kennedy, 1988]) is unlikely. This is not too surprising since peptone based

Table 8. Experimental conditions for the beginnings of a standardized buccal epithelial cell adhesion assay for *Candida albicans*[*]

Assay parameter	Specific condition
Assay parameters	1. Temperature: 37°C 2. Yeast: Buccal ratio (100: 1) 3. Reaction time: 1 hour 4. Assay buffer: Buffered KCl at pH 6.8[1]
Epithelial cells	1. Source 5 to 10 healthy adults (male & female) 2. Buffered KCl at pH 6.8
Yeast cells	1. *Candida albicans* AK785 (as the reference strain) 2. Growth media: LBC + 50 mM glucose YNB + 500 mM galactose 3. Growth phase: early stationary 4. Growth temperature: 37°C

[*]Designed from criteria described by Kennedy (1988).

[1]Buffered KCl is a solution that mimics the ionic composition of saliva (Gibbons *et al.*, 1985).

media are highly complex and undefined. Major differences in the chemical composition of peptones and "Sabouraud's medium" from laboratory to laboratory, from manufacturer to manufacturer, and from lot to lot of medium from the same manufacturer has been noted by Odds and collegues (Odds, 1985; Odds et al., 1978). Moreover, the use of complex and undefined media can modify several cell surface properties (e.g., cell surface hydrophobicity) which may directly or indirectly modify adhesion of C. albicans to epithelial and plastic surfaces. Therefore, it is suggested that the medium of Lee et al. (Lee et al., 1975) and yeast nitrogen base (Difco Laboratories) supplemented with 500 mM galactose (McCourtie & Douglas, 1981) be used because both media are chemically defined and produce cells of C. albicans that are highly adhesive. Furthermore, both media apparently yield Candida cells that have different adhesins and different cell surface characteristics. Early stationary phase cells are also suggested because stationary phase cells have been shown to adhere more readily than logarithmic phase cells (Kimura & Pearsall, 1978; King et al., 1980; McCourtie & Douglas, 1981 & 1984; Sandin et al., 1982) and growth should be at 37^{o}C (shaken at 100 rpm) (Kennedy, 1988).

Several other factors which are important to the development of a standardized buccal cell adhesion assay have been described in detail elsewhere (Kennedy, 1988). Nevertheless, further steps toward the standardization of

Candida adhesion assays will be necessary if the exact nature of C. albicans adhesion to BECs and other surfaces is to be understood. Because there are few similarities between the results of different investigators, the assay described in Table 8 was designed to incorporate what is presently known regarding C. albicans adhesion to BECs and to similate the environment of the oral cavity (e.g., the assay medium was designed to mimic the ionic composition and pH of saliva [Gibbons et al., 1985]). Given that this adhesion assay is probably incorrect in some respects, its use could lead to the formulation of incisive questions to be addressed in the future as well as to help determine which experimental parameters best mimic the interaction of C. albicans with BECs as it occurs in vivo. Therefore, it is urged that this assay be adopted for use with C. albicans, even if only to determine which experimental conditions are to be kept and which are to be changed.

GASTROINTESTINAL COLONIZATION BY CANDIDA ALBICANS

The survival, implantation, and dissemination of Candida albicans and other Candida species from the GI tract appear to be influenced by a number of factors. The presence of an intact and functioning indigenous microflora, however, is probably the most important deterrent to whether C. albicans can persist in the gut and at what population levels. Several studies, in fact, have shown that intestinal bacteria are inhibitory to

Candida in vitro or in the gut of experimental animals containing a diflora of *C. albicans* and some single bacterial species (e.g., *Escherichia coli*) (Balish & Phillips, 1966b; Hummel *et al.*, 1973 & 1975; Isenberg *et al.*, 1960; Nishikawa *et al.*, 1969; Ozawa *et al.*, 1979; Paine, 1958). Nevertheless, although these early studies suggested that intestinal bacteria play an important role in controlling *C. albicans* in the gut, they have led to several erroneous conclusions regarding the identity of the organisms and mechanisms responsible for the suppression of *Candida* in the GI tract. This is probably due to the use of inappropriate experimental models that do not reflect interactions between *Candida* and intestinal bacteria as they occur *in vivo* (Kennedy & Volz, 1985a). Therefore, the present studies examined the mechanisms which regulate *C. albicans* populations *in vivo*, and describes the suppression of *Candida* not by a single bacterial species but by an entire complex intestinal microflora. In addition, the beginnings of a general theory to explain the regulation of *Candida* population levels in the gut is put forth.

As was described above, *C. albicans* and other fungi are able to readily colonize the GI tract of germfree and antibiotic-treated animals, whereas similar colonization of conventional animals or man is difficult to achieve. This in addition to numerous reports in the literature showing a high correlation between *Candida* infections and

antibiotic therapy (Odds, 1988; Rippon, 1988), has led to the now commonplace assumption that the incidence and severity of alimentary tract colonization by C. albicans, as well as pathologic consequences that can be associated with this colonization, is somehow controlled by antagonistic interactions exerted by the numerous microbial species that normally inhabit the gut. As mentioned already, several studies support this hypothesis and have shown that certain intestinal bacteria are inhibitory to both in vitro growth and GI colonization by Candida and other fungi (Odds, 1988). Unfortunately, however, most of the studies to date, although supporting this hypothesis, cannot be relied upon to reflect interactions as they normally occur in the GI tract. Nevertheless, although there may be little similarity between the inhibitory mechanisms observed in simple in vitro studies and those found in the intestine, these and the data described in this dissertation do suggest that the suppression of Candida in vivo is likely to be the result of the interdependence of a complex set of interactions. Moreover, these data further suggest that these interactions are probably regulated, collectively, by a complex indigenous microflora, and not to one or even a few bacterial species as has been suggested previously (see Kennedy & Volz, 1985a).

While it seems obvious that the normal flora is clearly important in the suppression of gut colonization

and dissemination by C. albicans, the data presented here also help to clear up previous contradictory results reported in the literature regarding the effects of the "normal" intestinal microflora on GI colonization by this fungus. Balish & Phillips (1966 a&b; Phillips & Balish, 1966) in their early studies, for instance, had reported that C. albicans established in "large numbers" in the gut of both germfree and conventional animals, whereas DeMaria et al. (1976) and Helstrom & Balish (1979) in later experiments found that conventional animals were significantly more resistant to intestinal colonization than antibiotic-treated and germfree animals, respectively. Such discrepancies, however, may be due to the animals selected for study, since many strains of mice are unsuitable for research on intestinal floral interactions because they lack a true indigenous microflora (see Kennedy & Volz, 1985b).

Historically, the study of interactions between Candida and the indigenous intestinal microflora have included, with rare exceptions, two general approaches. The first type of approach is to estimate Candida population levels in the presence of intestinal bacteria (both in vivo and in vitro). The second includes determining how well Candida grows in a milieu after intestinal bacteria have grown in it first and then were removed or measuring Candida growth after certain chemicals have been added to the culture. However,

because of a number of accompanying technical difficulties, each of these methods is somewhat limited and can be misleading at times. This suggests that a combination of both types of studies may be necessary to accurately describe the survival, implantation and dissemination of C. albicans from the GI tract. Moreover, because of the complexity of the interactions between Candida and the indigenous microflora, it is important that models that simulate, as closely as possible, the events as they occur in vivo be used if meaningful results are to be obtained (Freter, 1983).

The anaerobic continuous-flow (CF) culture model of the ecology of the large intestinal flora described here meets these stringent requirements in that it reproduces a number of interactions as they occur in vivo (Freter et al. 1983b). For instance, mixed populations of mouse cecal bacteria in CF cultures were maintained in the same proportions as in the mouse large intestine. Furthermore, the bacterial populations in CF cultures were able to resist implantation by "invader" organisms (e.g., Clostridium difficle or Escherichia coli) and reduced these populations to levels similar to those found after being fed to conventional mice (Freter et al., 1983 a&b; Wilson & Freter, 1986). Moreover, it was found that C. albicans was eliminated from CF cultures of mouse cecal flora at a rate indistinguishable from that of the mouse cecum of conventional adult mice. A number of other

similarities between interactions involving indigenous and invader organisms, and of the ecosystems themselves, have been described by Freter (1983). Nevertheless, because of the similarities of interactions between C. albicans and bacteria in CF cultures and in the gut, this model was used to elucidate the bacterial mechanisms that control Candida in the GI tract.

These studies strongly support the hypothesis that the suppression of Candida in the gut is the result of a complex series of interactions that is regulated by an equally complex "group" of bacteria (Kennedy & Volz, 1985a). Although several distinct mechanisms appear to collectively regulate the survival, implantation and dissemination of C. albicans and other fungi from the GI tract (Kennedy & Volz, 1985a), these tend to fall into two general mechanisms: (i) antagonizing the growth of C. albicans and (ii) inhibiting the association of Candida with the intestinal mucosa. These have been summarized in Table 9.

It is interesting to note from this table that a number of mechanisms are inhibitory to Candida. Discussions in the early literature of the possible inhibitory mechanisms which suppress Candida have suggested that anaerobiosis or competition for nutrients were the most important factors (Isenberg et al., 1969; Paine, 1958). However, in studies using CF cultures it was found that anaerobic conditions did not prolong the

Table 9. Mechanisms by which the intestinal microflora inhibits *Candida albicans* colonization and dissemination from the gastrointestinal tract

Type	Mechanism
I	Inhibition of mucosal association:
	- formation of thick layers of bacteria that inhibit penetration into the mucosa
	- competition for adhesion sites
	- production of inhibitory substances (e.g., SCFA & DCBA)
	- increased rate of disassociation
	- modification of metabolic activity of *C. albicans* so that adhesins or digestive enzymes could not be produced
II	Regulation of the *Candida* population size:
	- anaerobic condition of the gut
	- production of inhibitory substances (e.g., SCFA, DCBA & H_2S)
	- competition for limiting nutrients
	- restricting substrate availability
	- prolonging the lag phase and doubling time

lag phase of C. albicans as did CF culture filtrates, indicating that other inhibitory mechanisms were also operating to suppress the growth of Candida. Moreover, the inhibitory effect on growth in CF culture filtrates could not be reversed by adding carbon or nitrogen sources, vitamins, or trace nutrients (individually or collectively) to CF culture filtrates and incubating the resulting cultures (under varying pHs) aerobically. Therefore, the suppression of C. albicans by intestinal bacteria was not due solely to depletion of nutrients and anaerobiosis, but appeared to be due to a combination of these factors in addition to the production of inhibitory substances as a result of the metabolic activity of intestinal anaerobes.

Several breakdown products from the large number of primary dietary or host-derived substrates in the gut may antagonize Candida species, as might a number of substances synthesized by the microflora itself. The most likely inhibitory substances, however, would probably be those that are produced by several bacteria in the gut and, therefore, those that are produced in the highest concentrations. One such set of substances may be the short-chain fatty acids (SCFAs). These are produced in relatively high concentrations by intestinal anaerobes (Argenzio, 1981), and it was found that both the total and individual levels of SCFAs present in CF cultures coincided with the quantity of acids found in the large

intestine of conventional mice or human feces used to establish the CF cultures. Because of this, because much has been speculated about the role of SCFAs in the control of the intestinal microflora (Argenzio, 1981; Bergeim et al., 1941; Levinson, 1973) and since SCFAs have been shown to be inhibitory to Fleischman's yeast (i.e., Saccharomyces cerevisiae) (Bergeim, 1941; Bergeim et al., 1941), these acids were tested for inhibitory activity against C. albicans. These studies indicated that at normal concentrations SCFAs were inhibitory to Candida growth, prolonging both the lag phase and doubling times. Further comparison between minimal inhibitory concentrations for individual acids and "gut levels", or levels found in CF cultures, showed that both acetic and butyric acid could antagonize Candida growth at the levels found in the GI tract. A similar argument could be made to support the role of deconjugated bile acids (DCBAs) in the suppression of Candida in the gut (Kennedy & Volz, 1985a). For instance, deconjugated bile acids have also been shown to antagonize Candida growth and to induce morphologic changes in the fungus when grown in vitro (Marshall et al., 1987; Kennedy, unpublished data). As will be discussed below, SCFAs and DCBAs have also been found to inhibit the adhesion of C. albicans to intestinal mucosa (Kennedy & Volz, 1985a).

It is likely that there are several substances which may antagonize the growth of Candida in the gut, however,

Freter (1983) has suggested that it is theoretically impossible to account for the total suppression of a sensitive microorganism in the gut solely on the basis of the production of growth inhibitors. If this were the case, C. albicans would be eliminated if too much inhibitor were present. Conversely, when too little inhibitor were present the Candida population would increase until it became limited by some other mechanism (Freter, 1983). Although it has been reported that Candida population levels do show some increases or decreases from day to day in experimental animals (Ekenna & Sherertz, 1987), it still may be argued that other inhibitory mechanisms are also involved with the regulation of Candida populations in the gut. For one thing, depending upon an individual's eating habits and the type of foodstuffs ingested at different meals, there must be fluctuations in the production of growth inhibitors. Moreover, studies with CF cultures have shown that C. albicans can adapt itself and become resistant to certain "toxic" substances when it is implanted simultaneously with the intestinal microflora.

The finding that Candida, although replicating (albeit very slowly), could not persist in CF cultures of mouse cecal flora was probably due to a lack of adhesion sites or the inability to attach to the glass culture vessel. Similar studies performed in antibiotic-treated mice showed that C. albicans populations predominated

initially in the lumen but that mucosal-associated populations predominated after the intestinal microflora reestablished. Thus the adhesion and association of Candida with the GI mucosa may be an important determinant to colonization, since mucosal association would probably allow C. albicans to remain in the gut even at a severely depressed growth rate (Kennedy, 1988). Furthermore, it may also be that colonization of the mucosa would provide a more sheltered site from growth inhibitors than the lumen. The ability of the indigenous microflora to inhibit the association of Candida with the intestinal mucosa, then, also represents an important defense mechanism that inhibits Candida colonization and dissemination from the GI tract. This seems especially true when considering that the first step in dissemination from the GI tract must be association with the mucosa.

Although the dynamics of lumenal and mucosal-associated C. albicans populations have not been completely defined, the importance of three distinct but interdependent steps in the association and passage of Candida through the mucosa have been implicated (Kennedy et al, 1987). These include: (i) contact with the surface of the mucus gel, (ii) penetration or trapping in the mucus gel, and (iii) adhesion and penetration of the epithelial surface proper. The first step in this process is probably an entirely random event, whereas the latter steps are not. The latter steps apparently involve the

interaction of specific adhesin-receptor binding and enzymatic modification of the mucus gel and epithelial surface for attachment to and penetration of GI mucosa (Cole et al., 1988; Kennedy et al., 1987). Complicating this is the finding that C. albicans could associate with the mucosa by at least five distinct mechanisms. These included: adhesion to epithelium, adhesion to mucus, co-adhesion to adherent fungi, co-adhesion to adherent bacteria, and entrapment in the mucus gel overlying the epithelium (Kennedy et al., 1987). The physicochemical bases and ecological role each of these mechanisms may play in GI colonization by Candida have been described in detail elsewhere (Kennedy, 1988).

Despite all of this, the dense layers of bacteria lining the mucus gel do appear to play an important role in the resistance to GI colonization and dissemination by Candida (Kennedy & Volz, 1985a). It has been noted that the first step in mucosal association, whether it is for Candida or any microorganism, must be the penetration of the mucus gel (Freter, 1982). In infant mice, which lack a complete bacterial flora including the dense bacterial populations in the mucus gel (Davis et al., 1973; Schaedler et al., 1965), C. albicans can readily associate with and pass through the mucosa to initiate systemic infection (Field et al., 1981; Pope et al., 1979). Similarly, in adult mice treated with certain antibiotics to eliminate wall-associated populations of indigenous

bacteria, C. albicans was able to associate with the GI mucosa and spread to visceral organs (Kennedy & Volz, 1983 & 1985b). In contrast, it was shown that intestinal tissues that possessed an indigenous wall-associated microflora strongly inhibited mucosal association (in vitro and in vivo) and dissemination of C. albicans from the GI tract (Kennedy & Volz, 1985a). Mucosal association by Candida was also found to be inhibited by a number of mechanisms (Kennedy & Volz, 1985a). For example, mucosal association by C. albicans was blocked by competing for adhesion sites and physically blocking the larger yeast cells from penetrating into the mucus gel. In addition, data obtained from "adhesion assays" and examination of GI mucosa obtained from experimental animals challenged with C. albicans, suggested that the mucosa-associated flora inhibited Candida cells from penetrating deep into the mucus gel (Kennedy & Volz, 1985a). Moreover, the rate of disassociation from intestinal tissues by C. albicans from antibiotic-treated animals was nearly three times that of untreated animals (Kennedy & Volz, 1985a). Nearly 75% of the Candida cells that had associated with the cecal mucosa of untreated adult animals were removed within 1 h, whereas only about 30% of the Candida cells were removed in the same time from animals treated with antibiotics (Kennedy & Volz, 1985a).

Finally, certain chemical factors produced in the gut by the metabolic activity of the normal flora can inhibit

the association of C. albicans with intestinal mucosa (Kennedy & Volz, 1985a). SCFAs and DCBAs, for instance, significantly reduced mucosal association of Candida cells in an adhesion assay that was performed under anaerobic conditions similar to that found in the gut (Kennedy & Volz, 1985a). Since environmental parameters are known to influence Candida adhesion to vaginal and buccal epithelial cells (Lee & King, 1983), it follows that modification of the GI environment by intestinal bacteria also modified attachment of C. albicans to intestinal mucosa (Kennedy, 1988). Although the exact nature of this inhibition by SCFAs and DCBAs has not been elucidated, it has been suggested that these substances may have reduced the mucosal association of Candida by modifying Candida adhesins or mucosal receptors, or both (Kennedy & Volz, 1985a). Alternatively, it may also be that these chemicals alter one or more physicochemical factors (e.g., the size of the double diffusion layer or the surface charge of yeast cells) that may cause a reduction in Candida adhesion (Kennedy, 1988). Nonetheless, a correlation between the drop in the concentration of SCFAs and DCBAs in the intestinal tract and an increase in mucosal association by C. albicans following antibiotic treatment has clearly been shown (Kennedy & Volz, 1985a).

Several lines of evidence suggest that the anaerobic populations, which dominate the intestinal flora (Moore, 1977), normally suppress Candida in the gut (Kennedy &

Volz, 1985a). Although one recent publication suggests that the aerobic portion of the flora may also be involved with suppression of C. albicans in the gut (Ekenna & Sherertz, 1987), the overwhelming evidence from both patient and animal studies indicate that resistance to colonization and dissemination by Candida is due to intestinal anaerobes. For instance, treatment of experimental animals with antibiotics that decreased the total populations of strictly anaerobic bacteria in the cecum, allowed an increase in facultative bacteria and promoted C. albicans adhesion, colonization and dissemination from the GI tract (Kennedy and Volz, 1983 & 1985 a&b). In contrast, colonization and dissemination from the GI tract by Candida could not be induced by antibiotics that were active against aerobes or facultative bacteria but were sparing of the anaerobes (Andermont et al., 1983; Wingard et al., 1980). Studies in infant mice, furthermore, have shown that C. albicans to maintain a stable population of about 10^3 to 10^4 per gram in the cecum and large intestine of the mice for the first two weeks of life, after which Candida counts decline to undetectable levels by four weeks (Field et al., 1981). The timing of elimination of C. albicans in infant mice, then, coincides with the acquisition and increase of populations of anaerobic bacteria, previously shown to occur during the second and third weeks after birth (Davis et al., 1973; Lee & Gemmelle, 1972; Lee et

al., 1971; Schaedler et al., 1965). It is also interesting to note that the increase in SCFAs and DCBAs in the large intestine also coincides with the acquisition of intestinal anaerobes (Lee & Gemmell, 1972). Other evidence for the role of intestinal anaerobes in the regulation of Candida in the GI tract has been reviewed by Odds (1988). The studies described herein of the role of intestinal microflora in the control of Candida populations in appropriate models, therefore, may well clarify inconsistencies in the literature and indicate productive avenues for regulating GI colonization by Candida in such a way that the beneficial effects are retained and the detrimental effects are eliminated.

LITERATURE CITED

LITERATURE CITED

Abdelghaffar AA, Russell C: Role of strain difference and tetracycline on production of subcutaneous lesions by Candida albicans in experimental rats. Sabouraudia 17:210-217.

Anderson JD, Gillespie WA, Richmond MH: Chemotherapy and antibiotic resistance transfer between enterobacteria in the human gastrointestinal tract. J Med Microbiol. 6:461-473, 1973.

Anderson ML, Odds FC: Adherence of Candida albicans to vaginal epithelial: significance of morphological form and effect of ketoconazole. Mykosen 28:531-540, 1985.

Andermont A, Raiband P, Tancrede C: Effect of erythromycin on microbial antigonism: a study in gnotobiotic mice associated with a human fecal flora. J Infect Dis. 148:579-587, 1983.

Argenzio, RA. Short-chain fatty acids and the colon. Digest Dis Sci 26:97-99, 1981.

Baine WB, Koenig MG, Goodman JS: Clearance of Candida albicans from the bloodstream of rabbits. Infect Immun 10:1420-1425, 1974.

Bak AL, Stenderup A: DNA homology in yeasts: genetic relatedness within the genus Candida. J Gen Microbiol 59:21-30, 1969.

Balish E, Balish MJ, Salkowski CA, Lee KW, Bartizal KF: Colonization of congenitally athymic gnotobiotic mice by Candida albicans. Appl Envirn Microb 47:647-652, 1984.

Balish E, Phillips AW: Growth, morphogenesis and virulence of Candida albicans after oral inoculation in the germ-free and conventional chick. J Bacteriol 91:1736-1743, 1966.

Balish E, Phillips AW: Growth and virulence of Candida albicans after oral inoculation in the chick with a monoflora of either Escherichia coli or Streptococcus faecalis. J Bacteriol 91:1744-1749, 1966.

Balish E, Shih CN, Yale CE, Mandel AD: Effect of a prolonged stay in a locked environment on the microbial flora in dogs. Aerospace Med 45:1248-1254, 1974.

Ballou C: Structure and biosynthesis of the mannan component of yeast cell envelope. Adv Microbiol Physiol 14:93-158, 1976.

Barnes JL, Osgood W, Lee JC, King RD, Stein JH. Host-parasite interactions in the pathogenesis of experimental renal candidiasis. Lab Invest 49:460-467, 1983.

Barrett-Bee K, Hayes Y, Wilson RG, Ryley JF: A comparison of phospholipase activity, cellular adherence and pathogenicity of yeast. J Gen Microbiol 131:1217-1221, 1985.

Bartnicki-Garcia S: Fungal cell wall composition, in Laskin AI, Lechevalier HA (eds.) Handbook of Microbiology Vol. II: Microbial Composition. CRC Press, Cleveland, pp 201-214, 1973.

Beneke ES, Rogers AL: Medical Mycology Manual. Burgess Publishing Company. Minneapolis, 1980.

Bergeim O: Toxicity of intestinal volatile fatty acids for yeast and Esch. coli. J Infect Dis 66:222-234, 1940.

Bergeim O, Hanszen AH, Pincussen L, Weiss E: Relation of volatile fatty acids and hydrogen sulphide to the intestinal flora. J Infect Dis 66:155-166, 1941.

Bianchi DE: The lipid content of cell walls obtained from juvenile, yeast like and filamentous cells of Candida albicans. Antonie van Leeuwenhoek. J Microbiol Serol 33:324-332, 1967.

Bishop CT, Blank F, Gardner P: The cell wall polysaccharides of Candida albicans, glucan, mannan and chitin. Can J Chem 38:869-881, 1960.

Blyth W: Host-parasite relationships in experimental moniliasis. I. Candida albicans. Mycopathologia 10:262-282, 1959.

Bodey GP, Rodriguez V: Infection in cancer patients on a protected environment-prophylatic antibiotic program. Am J Med 59:497-504, 1975.

Braun P, Calderone R: Chitin synthesis in Candida albicans: Comparison of yeasts and hyphal forms. J Bacteriol 133:1472-1477, 1978.

Brawner DL, Cutler JE. Changes in surface topography of Candida albicans during morphogenesis. Sabouraudia: J Med Vet Mycol 23:389-393, 1985.

Brawner DL, Cutler JE. Variability in expression of cell surface antigens of Candida albicans during morphogenesis. Infect Immun 51:337-343, 1986.

Cabib E, Roberts R, Bowers B: Synthesis of yeast cell wall and its regulation. Ann Rev Biochem 51:763-793, 1982.

Calderone RA, Lehrer N, Segal E: Adherence of Candida albicans to buccal and vaginal epithelial cells: Ultrastructural observations. Can J Microbiol 30:1001-1007, 1984.

Cantrell HR, Widra A: Experimental candidiasis in cortisone-treated mice. J Bacteriol 87:1532, 1964.

Carpenter AM: Studies on Candida I. Identification of 100 yeastlike fungi isolated from children. Am J Clin Path 25:98-101, 1955.

Cassone A: Improved visualization of wall structure in Saccharomyces cerevisiae. Experientia 29:1302-1309, 1973.

Cassone A, Kerridge D, Gale EF: Ultrastructural changes in the wall of Candida albicans following cessation of growth and their possible relationship to the development of polyene resistance. J. Gen Microbiol 110:339-349, 1979.

Cassone A, Mattia E, Boldrini L: Agglutination of blastospores of Candida albicans by Concanavalin A and its relationship with the distribution of mannan polymers and ultrastructure of the cell wall. J Gen Microbiol 105:263-273, 1978.

Cassone A, Simonette N, Strippoli V: Ultrastructural changes in the wall during germ-tube formation from blastospores of C. albicans. J Gen Microbiol 77:417-426, 1973.

Caugant DA, Levin BA, Selander RK: Genetic diversity and temporal variation in the E coli population of a human host. Genetics 98:467-490, 1981.

Cawson RA, Rajasingham KC: Ultrastructural features of the invasive phase of C. albicans. Br J Dermatol 87:435-443, 1972.

Centeno A, Davis CP, Cohen MS, Warren MM: Modulation of Candida albicans attachment to human epithelial cells by bacteria and carbohydrates. Infect Immun 39:1354-1360, 1983.

Chaffin LW, Stocco DM: Cell wall proteins of Candida albicans. Can J Microbiol 29:1438-1444, 1983.

Chattaway FW, Bishop R, Holmes MR, Odds FC, Barlow AJE: Enzyme activities associated with carbohydrate synthesis and mycelial forms of Candida albicans. J Gen Microbiol 75:97-109, 1973.

Chattaway FW, Holmes MR, Barlow AJE: Cell wall composition of the mycelial and blastospore forms of Candida albicans. J. Gen Microbiol 51:367-376, 1968.

Chattaway FW, Shenolikar S, O'Reilly J: Changes in the cell surface of the dimorphic forms of Candida albicans by treatment with hydrolytic enzymes. J Gen Microbol 95:335-347, 1976.

Cheng KJ, Irvin RT, Costerton JW: Autochthonous and pathogenic colonization of animal tissues by bacteria. Can J Microbiol 27:461-490, 1981.

Chiew YY, Shepherd MG, Sullivan PA: Regulation of chitin synthesis during germ-tube formation in Candida albicans. Arch Microbiol 125:97-104, 1980.

Clark JD: Influence of antibiotics or certain intestinal bacteria on orally administered Candida albicans in germ-free and conventional mice. Infect Immun 4:731-737, 1971.

Cohen R, Roth FJ, Delgado E, Aheard DG, Kalser MH: Fungal flora of the normal human small and large intestine. New Eng J Med 280:638-641, 1969.

Cole GT, Seshan KR, Pope LM, Yancey RJ: Morphological aspects of gastrointestinal tract invasion by Candida albicans in the infant mouse. J Med Vet Mycol 26: 173-185.

Collins-Lech C, Kalbfleisch JH, Franson TR, Sohnle PG: Inhibition by sugars of Candida albicans adherence to human buccal mucosal cells and corneocytes in vitro. Infect Immun 46:831-834, 1984.

Cooper BH, Silva-Hutner M: Yeasts of medical importance, In Lennette EH (ed): Manual of Clinical Microbiology, 4th Edition. American Society for Microbiology, Washington, pp526-541, 1985.

Costerton JW, Irvin RT, Cheng K-J: The role of bacterial surface structures in pathogenesis. CRC Crit Rev in Microbiol 8:303-338, 1981.

Costerton JW, Marrie TJ, Cheng K-J: Phenomena of bacterial adhesion, in Savage DC, Fletcher M (eds): Bacterial Adhesion: Mechanisms and physiological significance. New York, Plenum Press, pp 3-43, 1985.

Cox F: Adherence of Candida albicans to buccal epithelial cells in children and adults. J Lab Clin Med 102:960-972, 1983.

Critchley IA, Douglas LJ. Isolation and partial characterization of an adhesin from Candida albicans. J Gen Microbiol 1987; 133:629-636.

Dastidar SG: Studies on the antigens of the genus Candida by gel diffusion. Indian J Med Res 56:247-249, 1968.

Davenport JC: The oral distribution of Candida in denture stomatitis. Br Dent J 129:151-156, 1970.

Davidson S, Brish M, Rubinstein E: Adherence of Candida albicans to buccal epithelial cells of neonates. Mycopathologia 85:171-173, 1984.

Davis CP, McAllister JS, Savage DC: Microbial colonization of the intestinal epithelium in suckling mice. Infect Immun 7:666-672, 1973.

DeMaria A, Buckley H, von Lichtenberg F: Gastrointestinal candidiasis in rats treated with antibiotics, cortisone, and azathroprine. Infect Immun 13:1761-1770, 1976.

Dewilde T, De Vroey C, Bosmans J, Hubens A, Vanbreuseghem R: Prevalence of Candida albicans in patients receiving total parenteral nutrition. Sabouraudia 20:169-171, 1982.

Derjagiun BV, Landau L: Theory of the stability of strongly charged lyophobic sols and of the adhesion of strongly charged particles in solutions of electrolytes. Acta Physiochim USSR 14:633-662, 1941.

Diamond RD, Krzesicki R: Mechanisms of attachment of neutrophils to Candida albicans pseudohyphae in the absence of serum, and of subsequent damage to pseudohyphae by microbial processes of neutrophils in vitro. J Clin Invest 61:360-369, 1978.

Djaczenko W, Cassone A: Visualization of new ultrastructural components in the cell wall of Candida albicans with fixatives containing tapo. J Cell Biology 52:186-190, 1971.

Domer JE: Intragastric colonization of infant mice with Candida albicans induces systemic immunity demonstrable upon challenge as adults. J Infect Dis 157:950-958, 1988.

Domer JE, Hector RF: Enhanced immune responses in mice treated with penicillin-tetracycline or trimethoprim-sulfamethoxazole when colonized intragastrically with Candida albicans. Antimicrob Ag Chemo. 31:691-697, 1987.

Douglas LJ: Adhesion of pathogenic Candida species to host surfaces. Microbiol Sci 2:243-247, 1985.

Douglas LJ: Surface composition and adhesion of Candida albicans. Biochem Soc Trans 13:982-984, 1985.

Douglas LJ, Houston JG, McCourtie J: Adherence of Candida albicans to human buccal epithelial cells after growth on different carbon sources. FEMS Microbiol Letters 12:241-243, 1981.

Douglas LJ, McCourtie J: Effect of tunicamycin treatment on the adherence of Candida albicans to human buccal epithelial cells. FEMS Microbiol Letters 16:199-202, 1983.

Doyle RJ, Nesbitt WE, Taylor KG: On the mechanism of adherence of Streptococcus sanguis to hydroxylapatite. FEMS Microbiol Letters 15:1-5, 1982.

Drasar BS, Hill MJ: Human intestinal flora. Academic Press, New York, 1974.

Drasar BS, Shiner M, McLeod GM: Studies on the intestinal flora. I. The bacterial flora of the gastrointestinal tract in healthy andachlorhydric persons. Gastroenterology 56:71-79, 1969.

Dubos R: Staphylococci and infection immunity. Am J Dis Child 105:643-645, 1963.

Dubos R, Schaedler RW, Costello R, Hoef P: Indigenous, normal, and antochothonous flora of the gastrointestinal tract. J Exp Med 122:67-76, 1965.

Dubos R, Schaedler RW, Stevens M: The effect of antibacterial drugs on the fecal flora of mice. J Exp Med 117:231-243, 1963.

Ekenna O, Sherertz RJ: Factors affecting colonization and dissemination of Candida albicans from the gastrointestinal tract of mice. Infect Immun 55:1558-1563, 1987.

Elorza MV, Mugui A, Sentandraeu R: Dimorphism in Candida albicans: Contribution of mannoproteins to the architecture of yeast and mycelial cell walls. J Gen Microbiol 131:2209-2216, 1985.

Elorza MV, Ricco H, Gozalbo D, Sentandreu R: Cell wall composition and protoplast regeneration in Candida albicans. Antonie van Leeuwenhoek 49:457-469, 1983.

English MP: The saprophytic growth of keratinophilic fungi on keratin. Sabouraudia 2:115-130, 1963.

Epstein JB, Truelove EL, Izutzu KT: Oral candidiasis: pathogenesis and host defense. Rev Infect Dis 6:96-106, 1984.

Eras P, Goldstein MJ, Sherlock P: Candida infection of the gastrointestinal tract. Medicine (Baltimore) 51:367-379, 1972.

Field LH, Pope LM, Cole GT, Guentzel MN, Berry LJ: Persistence and spread of Candida albicans after intragastric inoculation of infant mice. Infect Immun 31:783-791, 1981.

Finegold SM, Mathisen GE, George WL: Changes in human intestinal flora related to the administration of antimicrobial agents. In Hentge DJ (ed) Human intestinal microflora in health and disease. Academic Press, New York, 1983, pp 355-446.

Finegold SM, Sutter VL, Mathisen GE: Normal indigenous intestinal flora. In Hentge DJ (ed) Human intestinal microflora in health and disease. Academic Press, New York, 1983, pp 3-31.

Fletcher M: The question of passive versus active attachment mechanisms in non-specific bacterial adhesion, in Berkley RCW, Lynch JM, Rutter PR, Vincent B (eds): Microbial adhesion to surfaces. London, Ellis Horwood Limited, 1980, pp 197-210,

Franklin MA, Skoryna SC: Studies on natural gastric flora. I. Bacterial flora of fasting human subjects. Can Med Assoc J 95:1349-1355, 1966.

Freter R: Bacterial association with the mucus gel system of the gut. In: Schlessinger D (ed) Microbiology. American Society for Microbiology, Washington, pp 278-281, 1982.

Freter R: Experimental enteric Shigella and Vibrio infections in mice and guinea pigs. J Exp Med 104:411-418, 1956.

Freter R: Factors controlling the composition of the intestinal microflora. In Stiles HW, Loesche WJ, O'Brien TC (eds): Proceedings: microbial aspects of dental caries (a special supplement to Microbiology Abstracts). Washington, DC, vol 1, Information Retrieval, 1976, pp 109-120.

Freter R: Interdependence of mechanisms that control bacterial colonization of the large intestine. Microecol and Ther 14:89-96, 1984.

Freter R: Mechanisms that control the microflora in the large intestine. In Hentge DJ (ed) Human intestinal microflora in health and disease. Academic Press, New York, 1983, pp 33-54.

Freter R, Brickner H, Botney M, Cleven D, Aranki A: Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora. Infect Immun 39:676-685, 1983.

Freter R, Brickner H, Fekete J, O'Brien PCM, Vickerman MM: Testing of host-vector systems in mice. Recomb DNA Tech Bull 2:68-76, 1979.

Freter R, Brickner H, Fekete J, Vickerman MM, Carey KE: Survival and implantation of Escherichia coli in the intestinal tract. Infect Immun 39:686-703, 1983.

Freter R, Jones GW. Adhesive properties of Vibrio cholera: nature of the ineteraction with intact mucosal surfaces. Infect Immun 6:918-927, 1976.

Freter R, Jones GW: Models for studying the role of bacterial attachment in virulence and pathogenesis. Rev Infect Dis 5:5647-5658, 1983.

Freter R, O'Brien PCM, Macsai MS. Role of chemotaxis in the association of motile bacteria with intestinal mucosa: fitness and virulence of nonchemotactic Vibrio cholera mutants in infant mice. Infect Immun 34:222-233, 1981.

Freter R, O'Brien PCM, Macsai MS. Role of chemotaxis in the association of motile bacteria with intestinal mucosa: in vivo studies. Infect Immun 34:234-240, 1981.

Freter R, Stauffer E, Cleven D, Holdeman LV, Moore WEC: Continuous-flow cultures as in vitro models of the ecology of large intestinal flora. Infect Immun 39:666-675, 1983.

Gardiner R, Podgorski C, Day AW: Serological studies on the fimbriae of yeasts and yeast-like species. Botanical Gazette 143:534-541, 1982.

Garrison RG: Vegetative ultrastructure, in Arnold WN (ed): Yeast cell envelopes ultrastructure, Vol 1. Boca Raton, Florida, CRC Press, pp 139-160, 1981.

Geck P, Novak EK: Immunofluorescent studies on yeasts. Acta Microbiol Acad Sci Hung 14:13-22, 1967.

Gerhardt NB, Iglewski WJ: Alterations in the fecal flora of an individual frequently lacking coliforms. J Infect Dis 134:490-494, 1976.

Ghannoum MA, Burns GR, Elteen K, Radwin SS: Experimental evidence for the role of lipids in adherence of Candida spp. to human buccal epithelial cells. Infect Immun 54:189-193, 1986.

Ghannoum MA, Elteen KA: Correlative relationship between proteinase production, adherence and pathogenicity of various strains of Candida albicans. J Med Vet Mycol 24:407-413, 1986.

Ghannoum MA, Janini G, Khamis L, Radwan SS: Dimorphism-associated variations in the lipid composition of Candida albicans. J Gen Microbiol 32:2367-2375, 1986.

Giannella RA, Broitman SA, Zamcheck N: Gastric acid barrier to ingested microorganisms in man: Studies in vivo and in vitro. Gut 13:251-256, 1972.

Gibbons RJ, Etherden I, Peros W: Aspects of the attachment of oral streptococci to experimental pellicles, in Mergenhagen SE, Rosan B (eds): Molecular Basis of Oral Microbial Adhesion. American Society for Microbiology, Washington, pp 77-84, 1985.

Gopal P, Sullivan PA, Sheperd MG: Isolation and structure of glucan from regenerating spheroplasts of Candida albicans. J Gen Microbiol 130:1217-1225, 1984.

Gordon MA, Elliot JC, Hawkins TW: Identification of Candida albicans, other Candida species and Torulopsis glabrata by means of immunofluorescence. Sabouraudia 5:323-328, 1983.

Gotlieb-Jensen K, Anderson J: Occurrence of Candida in gastric ulcers: significance for the healing process. Gastroenterology 85:535-537, 1983.

Gracey M, Stone DE, Suharjono BS, Sunoto MD: Isolation of Candida species from the gastrointestinal tract in malnourished children. Am J Clin Nutri 27:345-349, 1974.

Guentzel MN, Cole GT, Pope LM: Animal models for candidiasis. In McGinnis MR (ed): Current Topics in Medical Mycology, Vol. 1. New York, Springer-Verlag, pp 57-116, 1985.

Guentzel MN, Herrera C: Effects of compromising agents on candidosis in mice with persistent infections initiated in infancy. Infect Immun 35:222-228, 1982.

Hartley CL, Clements HM, Linton KB: Effects of caphalexin, erythromycin and clindamycin on the aerobic gram-negative fecal flora in man. J Med Micro 11:125-135, 1978.

Hasenclever HF: The antigens of Candida albicans. Am Rev Resp Dis Suppl 92:150-158, 1965.

Hasenclever HF, Mitchell WO: Antigenic studies of Candida. I. Observation of two antigenic groups in Candida albicans. J Bacteriol 82:570-573, 1961a.

Hasenclever HF, Mitchell WO: Antigenic studies of Candida. III. Comparative pathogenicity of Candida albicans group A, group B and Candida stellatoidea. J Bacteriol 82:578-581, 1961b.

Hasenclever HF, Mitchell WO, Loewe J: Antigenic relationship of Candida albicans group A and group B to Candida stellatoidea and Candida tropicalis. J Bacteriol 82:547-577, 1961.

Hazen EL, Brown R, Mason A: Protective action of Fungicidin (Nystatin) in mice against virulence enhancing activity of Oxytetracycline on Candida albicans. Antibiot and Chemo 111:1125-1128, 1953.

Hazen KC, Plotkin BJ, Klimas DM: Influence of growth conditions on cell surface hydrophobicity of Candida albicans and Candida glabrata. Infect Immun 54:269-271, 1986.

Hector RF, Domer JE: Control of systemic spread of Candida albicans with ketoconazole in the stomachs of mice treated with cytarabine. J Infect Dis 147:946-950, 1983.

Hector RF, Domer JE: Mammary gland contamination as a means of establishing long-term gastrointestinal colonization of infant mice with Candida albicans. Infect Immun 38:788-790, 1982.

Helstrom PB, Balish E: Effect of oral tetracycline, the microbial flora and the athymic state on gastrointestinal colonization and infection of BALB/c mice with Candida albicans. Infect Immun 23:764-774, 1979.

Holdeman LV, Cato EP, Moore WEC (eds) Anaerobe Laboratory Manual, 4th ed Anaerobe Lab. Virginia Polytechnic Institute and State University, Blacksburg, 1977.

Howlett JA: The infection of rat tongue mucosa in vitro with five species of Candida. J Med Microbiol 9:309-316, 1976.

Howlett JA, Squier CA: Candida albicans ultrastructure: Colonization and invasion of oral epithelium. Infect Immun 29:252-2600, 1980.

Hummel RP, Maley MP, Miskell PW, Altemeier WA: Suppression of Candida albicans by Escherichia coli. J Trauma 15:413-418, 1975.

Hummel RP, Oestreicher EJ, Maley MP, Macmillan BG: Inhibition of Candida albicans by Escherichia coli in vitro and the germfree mouse. J Surg Res 15:53-58, 1973.

Huppert M, Cazin J, Smith H: Pathogenesis of Candida albicans infection following antibiotic therapy. III The effect of antibiotics on the incidence of Candida albicans in the intestinal tract of mice. J Bacteriol 70:440-447, 1955.

Isaacson RE: Pilus adhesins, in Savage DC, Fletcher M (eds): Bacterial Adhesion: Mechanisms and physiological significance. New York, Plenum Press, pp 307-336, 1985.

Isenberg H, Allerhand J, Berkman JJ, Goldberg D: Immunological and toxic differences between mouse virulent and mouse avirulent Candida albicans. J Bacteriol 86:1010-1016, 1963.

Isenberg HD, Pisano MA, Carito SL, Berkman JI: Factors leading to overt monilial disease. I. Preliminary studies of the ecological relationship between Candida albicans and intestinal bacteria. Antibiot Chemo 10:353-363, 1960.

Jenkins WM: Oral infections with Candida albicans. Scot Med J 18:192-200, 1973.

Jennison RF: Candida infections in a maternity hospital, in Winner HI, Hurley R (eds): Symposium on Candida Infections. London, Livingston, pp 44-51, 1966.

Jones GW: The attachment of bacteria to the surfaces of animal cells, in Reissig JL (ed): Microbial Interactions. Receptors and Recognition, series B vol 3. New York, Chapman and Hall, pp 139-176, 1977.

Jones GW: Adhesion to animal surfaces, in Marshall KC (ed): Microbial Adhesion and Aggregation. New York, Springer-Verlag, pp 71-84, 1984.

Jones GW: Mechanisms of the attachment of bacteria to animal cells, in Klug MJ, Reddy CA (eds): Current Perspectives in Microbial Ecology. Washington, DC, pp 136-143, 1984.

Jones GW, Abrams GD, Freter R. Adhesive properties of Vibrio cholera: adherence to isolated rabbit brush border membranes and hemagglutinating activity. Infect Immun 14:232-239, 1976.

Jones GW, Freter R. Adhesive properties of Vibrio cholera: nature of the interaction with isolated rabbit brush border membranes and human erythrocytes. Infect Immun 14:240-245, 1976.

Jones GW, Isaacson RE: Proteinaceous bacterial adhesins and their receptors. CRC Crit Rev Microbiol 10:229-260, 1983.

Jones GW, Richardson LA: The attachment to, and invasion of HeLa cells by Salmonella typhimurium: The contribution of mannose-sensitive and mannose-resistant haemagglutinating activities. J Gen Microbiol 127:361-370, 1981.

Jones GW, Richardson LA, Uhlman D: The invasion of HeLa cells by Salmonella typhimurium: Reversible and irreversible bacterial attachment and the role of bacterial motility. J Gen Microbiol 127:351-360, 1981.

Jonsen J: Serological studies in fungi: comparison of agglutinins and precipitins in anti-Candida sera. Acta Pathol Microbiol Scand 37:79-83, 1955.

Joshi SN, Garvin PJ, Sunwoo YC: Candidiasis of the duodenum and jejunum. Gastroenterology 80:829-833, 1981.

Kapica L, Blank F: Growth of Candida albicans on keratin as a sole source of nitrogen. Dermatol 115:81-105, 1957.

Karaev ZO, Velichko EV, Bykov VL: Main characteristics of the process of adhesion of Candida to human epithelial cells. Zhurnal Mikrobiologii, Epidemiologii I. Immunologii 7:59-61, 1986.

Kearns MJ, Davies P, Smith H: Variability of the adherence of Candida albicans strains to human buccal epithelial cells: Inconsistency of differences between strains related to virulence. Sabouraudia 21:93-98, 1983.

Kemp G, Solotorvsky M: Localization of antigens in mechanically disrupted cells of certain species of Candida and Torulopsis. J Immunol 93:305-314, 1964.

Kennedy MJ: Adhesion and association mechanisms of Candida albicans. In McGinnis MR (ed): Current Topics in Medical Mycology, Vol 2. New York, Springer-Verlag, pp 73-169, 1988.

Kennedy MJ: Role of motility, chemotaxis and adhesion in microbial ecology. Annals of New York Acad Science 506:260-273, 1987.

Kennedy MJ, Bajwa PS, Volz PA: Gastrointestinal inoculation of Sporothrix schenckii in mice. Mycopathologia 78:141-143, 1982.

Kennedy MJ, Volz PA: Dissemination of yeasts after gastrointestinal inoculation in antibiotic-treated mice. Sabouraudia 21:27-33, 1983.

Kennedy MJ, Volz PA: Ecology of Candida albicans gut colonization: Inhibition of Candida adhesion, colonization, and dissemination from the gastrointestinal tract by bacterial antagonism. Infect Immun 49:654-663, 1985a.

Kennedy MJ, Volz PA: Effect of various antibiotics on gastrointestinal colonization and dissemination by Candida albicans. Sabouraudia: J Med Vet Mycol 23:265-274, 1985b.

Kennedy MJ, Volz PA, Edwards CA, Yancey RJ: Mechanisms of association of Candida albicans with intestinal mucosa. J Med Microbiol 24:333-341, 1987.

Kessler G, Nickerson WJ: Glucomannan-protein complexes from cell walls of yeast. J Biol Chem 234:2281-2285, 1959.

Kimura LH, Pearsall NN. Adherence of Candida albicans to human buccal epithelial cells. Infect Immun 21:64-68, 1978.

Kimura LH, Pearsall NN: Relationship between germination of Candida albicans and increased adherence to human buccal epithelial cells. Infect Immun 28:464-468, 1980.

King RD, Lee JC, Morris AL. Adherence of Candida albicans and other Candida species to mucosal epithelial cells. Infect Immun 1980; 27:667-674.

Klotz SA, Drutz DJ, Zajic JE: Factors governing adherence of Candida species to plastic surfaces. Infect Immun 50:97-101, 1985.

Kobayashi K, Suginaka H: Comparison of cell wall and membrane proteins from eight Candida species. Sabouraudia: J Med Vet Mycol 22:341, 334, 1984.

Kolarova N, Masler L, Sikl D: Cell wall glycopeptides of Candida albicans serotypes A and B. Biochim Biophys Acta 328:221-227, 1973.

Koznin PJ, Taschdjian CJ: Enteric candidiasis: diagnosis and clinical considerations. Pediatrics 30:71-85, 1962.

Krause W, Matheis H, Wulf K: Fungaemia and funguria after oral administration of Candida albicans. Lancet i:598-599, 1969.

Kreger-van Rij NJW (ed): The yeasts a taxonomic study, 3rd Edition. Elsevier, 1984.

Kulkarni RK, Hollingsworth PJ, Volz PA: Variation in cell surface features of Candida albicans with respect to carbon sources. Sabouraudia 18:255-260, 1980.

Kuo-SC, Lampen JO: Tunicamycin - an inhibitor of yeast glycoprotein synthesis. Biochem Biophys Res Comm 58:287-295, 1974.

Lee A, Gemmelle E: Changes in the mouse intestinal microflora during weaning: role of volatile fatty acids. Infect Immun 5:1-7, 1972.

Lee A, Gordon J, Lee CJ, Dubos R: The mouse intestinal microflora with emphasis on the strict anaerobes. J Exp Med 133:339-351, 1971.

Lee JC, King RD: Adherence mechanisms of Candida albicans, in Schlessinger D (ed): Microbiology - 1983. Washington, DC, American Society for Microbioogy pp 269-272, 1983.

Lee JC, King RD: Characterization of Candida albicans adherence to human vaginal epithelial cells in vitro. Infect Immun 41:1024-1030, 1983.

Lee KW, Balish E: Effect of T-cells and intestinal bacteria on resistance of mice to candidosis. RES: J Reticuloendoth Soc 31:233-240, 1982.

Lee KW, Balish MJ, Balish E: Resistance of germfree nude and thymus-bearing mice to systemic and gastrointestinal candidosis. Recent advances in germfree research. Proceedings of the VIIth International Symposium on Gnotobiology. Sasaki S, Ozawa A, Hashimoto K (eds). Tokai Univ Press, Tokyo, pp 429-432, 1981.

Lee KL, Buckley HR, Campbell CC. An amino acid liquid synthetic medium for development of mycelial and yeast forms of Candida albicans. Sabouraudia 13:148-153, 1975.

Lehrer N, Segal E, Barr-Nea L. In vitro and in vivo adherence of Candida albicans to mucosal surfaces. Ann Microbiol 134:293-306, 1983.

Levison ME: Effect of colon flora and short-chain fatty acids on growth in vitro of Pseudomonas aeruginosa and Enterobacteriaceae. Infect Immun 8:30-35, 1973.

Liljemark WF, Gibbons RJ: Suppression of Candida albicans by human oral Streptococci in gnotobiotic mice. Infect Immun 8:846-849, 1973.

Locci R, Peters G, Pulverer G: Microbial colonization of prosthetic devices. IV. Scanning electron microscopy of intravenous catheters invaded by yeasts. Zentralbl Bakteriol Hyg (B) 173:419-424, 1981.

Loeb GI: The properties of nonbiological surfaces and their characterization, in Savage DC, Fletcher M (eds): Bacterial Adhesion: Mechanisms and physiological significance. New York, Plenum Press, pp 111-129, 1985.

Ludlam GB, Henderson JL: Neonatal thrush in maternity hospital. Lancet i:64-70, 1942.

Luft JH: Ruthenium red and ruthenium violet. II. Fine structural purification, methods for use for electron microscopy and localization in animal tissues. Anat Rec 171:369-416, 1971.

MacDonald F, Odds FC: Virulence for mice of a proteinase-secreting strain of Candida albicans and a proteinase-deficient mutant. J Gen Microbiol 129:431-438, 1983.

Maisch PA, Calderone RA: Adherence of Candida albicans to a fibrin-platelet matrix formed in vitro. Infect Immun 27:650-656, 1980.

Maisch PA, Calderone RA: Role of surface mannan in the adherence of Candida albicans to fibrin-platelet clots formed in vitro. Infect Immun 32:92-97, 1981.

Marples MJ: Oral candidiasis. Br J Dermatol 80:58-59, 1968.

Marrie TJ, Costerton JW: The ultrastructure of Candida albicans infections. Can J Microbiol 27:1156-1164, 1981.

Marshall KC: Interfaces in microbial ecology. Cambridge, MA, Harvard University Press, 1976.

Marshall KC (ed): Microbial adhesion and aggregation. Berlin, Springer-Verlag, 1984.

Marshall SE, Marples BA, Salt WG, Stretton RJ: Aspects of the effect of bile salts on Candida albicans. J Med Vet Mycol 25:307-318, 1987.

McCourtie J, Douglas LJ. Relationship between cell surface composition of Candida albicans and adherence to acrylic after growth on different carbon sources. Infect Immun 32:1234-1241, 1981.

McCourtie J, Douglas LJ. Relationship between cell surface composition, adherence and virulence of Candida albicans. Infect Immun 45:6-12; 1984.

McCourtie J, Douglas LJ. Extracellular polymer of Candida albicans: isolation, analysis and role in adhesion. J Gen Microbiol 1985a; 131:495-503.

McCourtie J, Douglas LJ. Unequal distribution of adhesins within populations of Candida albicans. FEMS Microbiol Lett 1985b; 27:111-115.

McCourtie J, MacFarlane TW, Samaranayake LP: Effect of chlorhexidine gluconate on the adherence of Candida species to denture acrylic. J Med Microbiol 20:97-104, 1985.

Meunier-Carpenter F, Kiehn TE, Armstrong D: Fungaemia in the immunocompromised host: changing patterns, antigenemia, high mortality. Am J Med 71:363-370, 1981.

Miles MR, Olsen L, Rogers A: Recurrent vaginal candidiasis: Importance of an intestinal reservoir. J Am Med Assoc 238:1836-1837, 1977.

Miller GR, Sarachek A: Absence of photoreactivating enzymes in Candida albicans, Candida stellatoidea and Candida tropicalis. Infect Immun 10:398-399, 1974.

Minagi S, Miyaka Y, Fugioka Y, Tsuru H, Suginaka H: Cell-surface hydrophobicity of Candida species as determined by the contact-angle and hydrocarbon-adherence methods. J Gen Microbiol 132:1111-1115, 1986.

Minagi S, Miyake Y, Inagaki K, Tsuru H, Suginaka H: Hydrophobic interaction in Candida albicans and Candida tropicalis adherence to various denture base resin materials. Infect Immun 47:11-14, 1985.

Miyake Y, Fujita Y, Minagi S, Suginaka H: Surface hydrophobicity and adherence of Candida to acrylic surfaces. Microbios 46:7-14, 1986.

Montes LF, Wilborn WH. Ultrastructure features of host-parasite relationship in oral candidiasis. J Bacteriol 96:1349-1356, 1968.

Montes LF, Wilborn WH. Fungus-host relationship in candidiasis. Arch Dermatol 1985; 121:119-124.

Montplaisir S, Nabarra B, Drouhet E: Susceptibility and resistance of Candida to 5-Fluorocytosine in relation to the cell wall ultrastructure. Antimicrob Agents Chemo 9:1028-1032, 1976.

Moore WEC: Anaerobes as normal flora: gastrointestinal tract. Metronidazole: Proceedings, Montreal, 1977, pp 222-228.

Moore WEC, Cato EP, Good IJ, Holdeman LV: The effect of diet on the human fecal flora. Banbury Report 7: Gastrointestinal Cancer: Endogenous Factors pp 11-23, 1981.

Moore WEC, Holdeman LV: The normal flora of 20 japanese Hawaiians. Appl Microbiol 27:961-979, 1974.

Muller HL, Kirchhoff G: Serologische typen von Candida albicans. Zentralbl Bakteriol Parasitenk Infektionskr Hyg Abt 210:114-121, 1969.

Murray IG, Buckley HR: Serological study of Candida species, in Winner HI, Hurley R (eds): Symposium on Candida Infections. London, Livingston, pp 44-51, 1966.

Myerowitz RL: Gastrointestinal and disseminated candidiasis; An experimental model in the immunosuppressed rat. Arch Pathol Lab Med 105:138-143, 1981.

Myerowitz RL, Pazin GJ, Allen CM: Disseminated Candidiasis: Changes in incidence, underlying diseases, and pathology. Am J Clin Path 68:29-38, 1977.

Nishikawa T, Hatano H, Ohnishi N, Sasake S, Nomura T: Establishment of Candida albicans in the alimentary tract of the germ-free mice and antagonism with Escherichia coli after oral inoculation. Jap J Microbiol 13:263-276, 1969.

Nystatin Multicenter Study Group: Therapy of candidal vaginitis: The effect of eliminating intestinal Candida. Am J Obstet Gynecol 155:651-655, 1986.

Odds FC. Biotyping of medically important fungi, in McGinnis MR (ed): Current Topics in Medical Mycology, vol. 1. Springer-Verlag, New York, pp 157-171, 1985.

Odds FC: Candida and Candidosis. Baltimore, University Park Press, 1988.

Odds FC, Gonzales A: Acid phosphatase levels in the genus Candida and their application to the taxonomy and identification of pathogenic Candida species. Sabouraudia 12:287-294, 1974.

Odds FC, Hall CA, Abbott AB. Peptones and mycological reproducibility. Sabouraudia 16:237-246, 1978.

Okubo Y, Honma Y, Suzuki S: Relationship between phosphate content and serological activities of the mannans of Candida albicans strains NIH A-207, NIH B-792, and J-1012. J Bacteriol 137:677-680, 1979.

Ozawa K, Yabu-Uchi K, Yamanaka K, Yamachita Y, Ueba K, Miwatani T: Antagonistic effects of Bacillus natto and Streptococcus faecalis on growth of Candida albicans. Microbiol Immunol 23:1147-1156, 1979.

Paerl HW: Influence of attachment on microbial metabolism and growth in aquatic ecosystems, in Savage DC, Fletcher M (eds): Bacterial adhesion: Mechanisms and physiological significance. New York, Plenum Press, pp 363-400, 1985.

Paine TF: The inhibitory actions of bacteria on Candida growth. Antibiotics Chemo 8:273-281, 1958.

Persi MA, Burnham JC, Duhring JL: Effects of carbon dioxide and pH on adhesion of Candida albicans to vaginal epithelial cells. Infect Immun 50:82-90, 1985.

Phillips AW, Balish E: Growth and invasiveness of Candida albicans in the germfree and conventional mouse after oral challenge. Appl Microbiol 14:737-741, 1966.

Phillips MW, Lee A: Microbial colonization of rat colonic mucosa following intestinal pertubation. Microbial Ecol 10:79-88, 1984.

Plaut AG, Gorbach SL, Nahas L, Weinstein L, Spanknebel G, Levitan R: Studies of intestinal microflora. III. The microbial flora of human small intestinal mucosa and fluids Gasteroenterology 53:868-873, 1967.

Ponton J, Jones JM: Analysis of cell wall extracts of Candida albicans by sodium dodecyl sulfate-polyacrylamide gel elecrophoresis and western blot techniques. Infect Immun 53:565-572, 1986a.

Ponton J, Jones JM: Identification of two germ-tube-specific cell wall antigens of Candida albicans. Infect Immun 54:864-868, 1986b.

Pope LM, Cole GT, Guentzel MN, Berry LJ: An experimental model of candidiasis in infant mice: Systemic infection following gastrointestinal colonization, in Kuttin ES, Baum GL (eds): Human and Animal Mycology. Amsterdam, Escerpta Medica, 1979, pp 71-74.

Pope LM, Cole GT, Guentzel MN, Berry LJ: Systemic and gastrointestinal candidiasis of infant mice after intragastric challenge. Infect Immun 25:702-707, 1979.

Pope LM, Cole GT: SEM studies of adherence of Candida albicans to the gastrointestinal tract of infant mice. Scan Electron Microsc 3:73-80, 1981.

Pope LM, Cole GT: Comparative studies of gastrointestinal colonization and systemic spread by Candida albicans and nonlethal yeast in the infant mouse. Scan Electron Microsc 4:1667-1676, 1982.

Poulain D, Hopwood V, Vernes A. Antigen variability of Candida albicans. Crit Rev Microbiol 12:223-271, 1985.

Poulain D, Tronchin G, Jouvert S, Herbant J, Biguet J: Architecture pariétale des blastospores de Candida albicans localization de composants chimiques et antigéniques. Ann Microbiol 132A:219-223, 1981.

Pugh D, Cawson RA: The cytochemical localization of phospholipase and lysophoslipase in Candida albicans. Sabouraudia 13:110-115, 1975.

Pugh D, Cawson RA: The cytochemical localization of acid hydrolases in four common fungi. Cell Mol Biol 22:125-132, 1977.

Pugh D, Cawson RA: The cytochemical localization of phospholipase in Candida albicans infecting the chick chorio-allantoic membrane. Sabouraudia 15:29-35, 1977.

Pugh D, Cawson RA: The surface layer of Candida albicans. Microbios 23:19-23, 1978.

Ray TL: Fungal infections in the immunocompromised host. Med Clin N Am 64:955-968, 1980.

Rebora A, Leyden JJ: Napkin (diaper) dermatitis and gastrointestinal carriage of Candida albicans. Brit J Derm 105:551-555, 1981.

Reinhart H, Muller G, Sobel JD: Specificity and mechanism of in vitro adherence of Candida albicans. Ann Clin Lab Sci 15:406-413, 1985.

Reiss E: Molecular immunology of mycotic and actinomycotic infections. New York, Elsevier, 1986.

Richardson MD, Smith H: Resistance of virulent and attenuated strains of Candida albicans to intracellular killing by human and mouse phagocytes. J Infect Dis 144:557-564, 1981.

Rippon JW: Medical Mycology: The pathogenic fungi and the pathogenic actinomycetes. WB Saunders Company, Philadelphia, 1988.

Rogers TJ, Balish E: Immunity to Candida albicans. Microb Rev 44:660-682, 1980.

Rossano F, Tufano MA: Effect of concanavalin A on the adhesiveness of Candida albicans to vaginal cells. Rass Med Sper 22:313-317, 1975.

Rotrosen D, Calderone RA, Edwards JE: Adherence of Candida species to host tissues and plastic surfaces. Rev Infect Dis 8:73-85, 1986.

Rotrosen D, Edwards JE, Gibson TR, Moore JC, Cohen AH, Green I: Adherence of Candida to cultured vascular endothelial cells: Mechanisms of attachment and endothelial cell penetration. J Infect Dis 152:1264-1274, 1985.

Rotrosen D, Gibson TR, Edwards JE: Adherence of Candida species to intravenous catheters. J Infect Dis 147:594, 1983.

Rutter PR, Vincent B: Physiochemical interactions of the substratum, microorganisms, and the fluid phase, in Marshall KC (ed): Microbial Adhesion and Aggregation. New York, Springer-Verlag, pp 21-38, 1984.

Rutter PR, Vincent B: The adhesion of microorganisms to surfaces: Physiochemical aspects, in Berkley RCW, Lynch

JM, Rutter PR, Vincent B (eds): Microbial adhesion to surfaces. London, Ellis Horwood Limited, pp 79-92, 1980.

Ryley JF: Pathogenicity of Candida albicans with particular reference to the vagina. J Med Vet Mycol 24:5-22, 1986.

Samaranayake LP, MacFarlane TW: An in vitro study of the adherence of Candida albicans to acrylic surfaces. Archs Oral Biol 25:603-609, 1980.

Samaranayake LP, MacFarlane TW: Factors affecting the in vitro adherence of the fungal oral pathogen Candida albicans to epithelial cells of human origin. Archs Oral Biol 27:869-873, 1982.

Samaranayake LP, MacFarlane TW: The effect of dietary carbohydrates on the in vitro adhesion of Candida albicans to epithelial cells. J Med Microbiol 15:511-517, 1982.

Samaranayake LP, MacFarlane TW: On the role of dietary carbohydrates in the pathogenesis of oral candidosis. FEMS Microbiol Lett 27:1-5, 1985.

Samaranayake LP, McCourtie J, MacFarlane TW: Factors affecting the in vitro adherence of Candida albicans to acrylic surfaces. Arch Oral Biol 25:611-615, 1980.

San-Blas G: The cell wall of fungal human pathogens: Its possible role in host-parasite relationships. Mycopathologia 79:159-184, 1982.

Sandin RL. Studies on cell adhesion and concanavalin A induced agglutination of Candida albicans after mannan extraction. J Med Microbiol 24:145-150, 1987a.

Sandin RL. The attachment to human buccal epithelial cells by Candida albicans: an in vitro kinetic study using concanavalin A. Mycopathologia 98:179-184, 1987b.

Sandin RL, Rogers AL. Inhibition of adherence of Candida albicans to human buccal epithelial cells. Mycopathologia 77:23-26, 1982.

Sandin RL, Rogers AL, Patterson RJ, Beneke ES. Evidence for mannose-mediated adherence of Candida albicans to human buccal epithelial cells in vitro. Infect Immun 35:79-85. 1982.

Saugar VK, Cortes JM, Self LW: Acid phosphatase production as an aid in rapid characterization of Candida species. Am J Med Technol 41:327-332, 1975.

Savage DC: Adherence of normal flora to mucosal surfaces. Beachey EH (ed) Bacterial Adherence, London, Chapman and Hall, pp 33-59, 1980.

Savage DC: Associations of indigenous microorganisms with gastrointestinal epithelial surfaces, in Hentges DJ (ed): Human intestinal microflora in health and disease. New York, Academic Press, pp 55-78, 1983.

Savage DC: Associations of indigenous microorganisms with gastrointestinal mucosal epithelia. Am J Clin Nutr 23:1495-1501, 1970.

Savage DC: Localization of certain indigenous microorganisms on the ileal villi of rats. J Bacteriol 97:1505-1506, 1969.

Savage DC: Microbial ecology of the gastrointestinal tract. Ann Rev Microbiol 31:107-133, 1977.

Sawai T, Hehre EJ: The distribution of transglucosyl-amylase among Candida yeasts. Antonie van Leeuwenhoek J Microbiol Serol 29:377-385, 1963.

Schaedler RW, Dubos R, Costello R: Association of germfree mice with bacteria isolated from normal mice. J Exp Med 122:77-83, 1965.

Schaedler RW, Dubos R, Costello R: The development of the bacterial flora in the gastrointestinal tract of mice. J Exp Med 122:59-66, 1965.

Schertwitz C, Martin R, Ueberberg H. Ultrastructural investigations of the formation of Candida albicans germtubes and septa. Sabouraudia 16:115-124, 1978.

Schwartz DS, Larsh HW: An effective medium for the selective growth of yeast or mycelial forms of Candida albicans: Biochemical aspects of the two forms. Mycopathologia 70:67-75, 1980.

Schwartz DS, Larsh HW: Comparative activities of glycolytic enzymes in yeast and mycelial forms of Candida albicans. Mycopathologia 78:93-98, 1982.

Sears HJ, Brownlee I: Further observations on the persistence of individual strains of Escherichia coli in the intestinal tract of man. J Bacteriol 63:47-57, 1952.

Sears HJ, Brownlee I, Uchigama JK: Persistence of individual strains of Escherichia coli in the intestinal tract of man. J Bacteriol 59:293-301, 1949.

Segal E, Lehrer N, Ofek I: Adherence of Candida albicans to human vaginal epithelial cells: Inhibition by amino sugars. Exp Cell Biol 50:13-17, 1982.

Segal E, Savage DC: Adhesion of Candida albicans to mouse intestinal mucosa in vitro: Development of the assay and test of inhibitors. J Med Vet Mycol 24:477-479, 1986.

Segal E, Soroka A, Schechter A: Correlative relationship between adherence of Candida albicans to human vaginal epithelial cells in vitro and Candidal vaginitis. Sabouraudia 22:191-200, 1984.

Segal E, Vardinon N, Schwartz J, Eylan E: Antigenic relationships between Candida albicans and various yeasts as reflected by immunoglobulin-class specificity. Pathol Microbiol 42:49-58, 1975.

Shecter Y, Landau JW, Dabrowa N: Comparative electrophoresis and numerical taxonomy of same Candida species. Mycologia 64:841-853, 1972.

Shedlofsky S, Freter R: Synergism between ecological and immunological control mechanisms of intestinal flora. J Infect Dis 129:296-303, 1974.

Shepherd MG, Poulter RTM, Sullivan PA: Candida albicans: Biology, genetics, and pathogenicity. Ann Rev Microbiol 39:579-614, 1985.

Shepherd MG, Surarit R, Gopal PK, Sullivan PA: Cell wall metabolism of C. albicans: B(13) and B(16) glucan synthesis, in Nombela C (ed). Microbial cell wall synthesis and autolysis. Elsevier Science Publishers, New York, pp 73-83, 1984.

Sikl D, Masler C, BAuer S: Mannan from the extracellular surface of Candida albicans Berkhout. Experientia 20:456, 1964.

Skerl KG, Calderone RA, Segal E, Sreevalsan T, Scheld WM: In vitro binding of Candida albicans yeast cells to human fibronectin. Can J Microbiol 30:221-227, 1984.

Slutsky B, Buffo J, Soll DR. High frequency switching of colony morphology in Candida albicans. Science 230:666-669, 1985.

Slutsky B, Staebell M, Anderson J, Risen L, Pfaller M, Soll DR. 'White-opaque transition': a second high-frequency switching system in Candida albicans. J Bacteriol 169:189-197, 1987.

Smail EH, Jones JM. Demonstration and solubilization of antigens expressed primarily on the surfaces of Candida albicans germ tubes. Infect Immun 45:74-81, 1984.

Smyth CJ, Jonsson P, Olsson E, Söderlind O, Rosengren J, Hjerten S, Wadstrom T: Differences in hydrophobic surface characteristics of porcine enteropathogenic Escherichia coli with or without K88 antigen as revealed by hydrophobic interaction chromatography. Infect Immun 22:462-472, 1978.

Sobel JD, Myers PG, Kaye D, Levison ME: Adherence of Candida albicans to human vaginal and buccal epithelial cells. J Infect Dis 143:76-82, 1981.

Spencer JF, Gorin PAJ: Systematics of the genus Candida Berkhout. PMR spectra of the mannans and mannose-containing polysaccharides as an aid in classification. Antonie van Leeuwenhoek J Microbiol Serol 35:33-44, 1969.

Stewert-Tull DES, Timperley WK, Horne CHW: Some immunochemical observations of the cell walls of yeasts. Sabouraudia 5:104-109, 1966.

Stone HH: Studies in the pathogenesis, diagnosis and treatment of Candida sepsis in children. J Pediatr Surg 9:127-133, 1974.

Stone HH, Geheber CE, Kolb LD, Kitchens WR: Alimentary tract colonization by Candida albicans. J Surg Res 14:273-276, 1973.

Stone HH, Kolb LD, Currie CH, Gebeher CE, Cuzzell JF: Candida sepsis: pathogenesis and principles of treatment. Ann Surg 179:697-711, 1974.

Sullivan PA, Yin CY, Molloy C, Templeton MD, Shepherd M: An analysis of the metabolism and cell wall composition of Candida albicans during germ tube formation. Can J Microbiol 29:1514-1525, 1983.

Sundstrom PM, Kenny GE. Characterization of antigens specific to the surface of germ tubes of Candida albicans by immunofluorescence. Infect Immun 43:850-855, 1984.

Sundstrom PM, Nichols EJ, Kenny GE. Antigenic differences between mannoproteins of germ tubes and blastospores of Candida albicans. Infect Immun 55:616-620, 1987.

Suzuki M, Fukazawa Y: Immunochemical characterization of Candida albicans cell wall antigens: Specific determinant of Candida albicans serotype A mannan. Microbiol Immunol 26:387-402, 1982.

Suzuki S, Sunayama H: Studies on the antigenic activities of yeasts. Jap J Microbiol 13:95-101, 1969.

Suzuki S, Usuyama M: Effect of Arthrobacter -mannosidase digestion on the immunochemical properties of two serotypes of Candida yeasts. Tohuku Yakka Diagaku Kenkyu Nempo 18:55-62, 1971.

Sweet CE, Kaufman L: Application of agglutinins for the rapid and accurate identification of medically important Candida species. Appl Microbiol 19:830-836, 1970.

Tadros TF: Particle-surface adhesion, in Berkley RCW, Lynch JM, Rutter PR, Vincent B (eds): Microbial adhesion to surfaces. London, Ellis Horwood Limited, pp 79-92, 1980.

Thomas E, Reddy KR: Nonhealing duodenal ulceration due to Candida. J Cin Gastro 5:55-58, 1983.

Thienpont D, vanCutsem J, Gough DA: Treatment of gastrointestinal candidosis in predisposed guinea pigs and in conventional mice with miconazole. Mykosen 21:417-424, 1978.

Tronchin G, Poulain D, Herbaut J, Biguet J. Localization of chitin in the cell wall of Candida albicans by means of wheat germ agglutinin. Fluorescence and ultrastructural studies. Eur J Cell Biol 26:121-128, 1981.

Tronchin G, Poulain D, Vernes A. Cytochemical and ultrastructural studies of Candida albicans. III. Evidence for modifications of the cell wall coat during adherence to human buccal epithelial cells. Arch Microbiol 139:221-224, 1984.

Tsuchiya T, Fukazawa Y, Kawakita S: Serological classificaiton of the genus Candida, in Tokyo: Research Committe on Candidiasis, Education Ministry of Japan: Studies on candidiasis in Japan, pp34-46, 1961.

Turner JR, Butler TF, Johnson ME, Gordee RS: Colonization of the intestinal tract of conventional mice with Candida albicans and treatment with antifungal agents. Antimicrob Agents Chemother 9:787-792, 1976.

Umenai T: Systemic candidiasis produced by oral Candida administration in mice. Tohoku J Exp Med 126:173-175, 1978.

Umenai T, Konno S, Ishida N: Systemic candidiasis by Candida albicans colonized in the gastrointestinal tract of mice. Experientia 35:1331-1332, 1979.

Umenai T, Konno S, Yamauchi A, Iimura Y, Fujimoto H: Growth of Candida in the upper intestinal tract as a possible source of systemic candidiasis in mice. Tohoku J Exp Med 130:101-102, 1980.

van der Waaij D, Berghuis de Vries JM, Lekkerkerk-van der Wees JEC: Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J Hyg 69:405-511, 1971.

Verwey EJW, Overbeek JTG: Theory of the stability of lyophobic colloids. Elsevier, Amsterdam, 1948.

Vudhichamnog K, Walter DM, Ryley HC: The effect of secretory immunoglobulin A on the in vitro adherence of the yeast Candida albicans to human oral epithelial cells. Arch Oral Biol 27:617-621, 1982.

Waid JS: Physiological and biochemical adjustment of fungi to their environment, an advanced treatise, in Ainsworth GC, Sussman AS (eds): The Fungi, Vol 3. Academic Press, New York, pp 298-301, 1978.

Walsh TJ, Merz WG: Pathologic features in the human alimentary tract associated with invasiveness of Candida tropicalis. Am J Clin Path 85:498-502, 1986.

White LO, Gibbs E, Newman HC, Richardson MD, Warren RC: Comparison of the growth of attenuated strains of Candida albicans in the kidneys of normal and cortisone-treated mice by chitin assay. Mycopathologia 67:173-177, 1979.

Wilborn WH, Montes LF: Scanning electron microscopy of oral lesions in chronic mucocutaneous candidiasis. JAMA 244:2294-2297, 1980.

Wilson KH, Freter R: Interaction of Clostridium difficile and Escherichia coli with microfloras in continuous-flow cultures and gnotobiotic mice. Infect Immun 54:354-358, 1986.

Wingard JR, Dick JD, Merz WG, Sanford GR, Saral R, Burns WH: Differences in virulence of clinical isolates of Candida tropicalis and Candida albicans in mice. Infect Immun 37:833-836, 1982.

Wingard JR, Dick JD, Merz WG, Sanford GR, Saral R, Burns WH: Pathogenicity of Candida tropicalis and Candida albicans after gastrointestinal inoculation in mice. Infect Immun 29:808-813, 1980.

Winner HI, Hurley R: Candida albicans. London, Churchill, 1964.

Yamaguchi H, Kanda Y, Iwata K: Biochemical properties of mitchondria from Candida albicans. Sabouraudia 9:221-230, 1971.

Yu R, Bishop C, Cooper F, Hasenclever H: Glucans from Candida albicans (serotype B) and from Candida parapsilosis. Can J Chem 45:2264-2267, 1967.

Yu R, Bishop C, Cooper F, Hasenclever H, Blank F: Structural studies of mannan from Candida albicans (serotype A et B), Candida parapsilosis, Candida stellatoidea, and Candida tropicalis. Can J Chem 45:2205-2211 , 1967.

Zubrzycki L, Spaulding EH: Application of the continuous-flow culture method to studies on the normal fecal flora. Bacteriol Proc , p 101, 1957.

Zubrzycki L, Spaulding EH: Studies on the stability of the normal human fecal flora. J Bacteriol 83:968-974, 1962.