AN EXPERIMENTAL STUDY OF THE INFECTION OF ORAL MUCOSA IN VITRO BY CANDIDA

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A Thesis submitted for the degree of Doctor of Philosophy of the University of London

OMH

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Poor text in the original thesis.

Some images distorted
An experimental study of oral mucosa in vitro infected with species of Candida - by Julie A. Howlett

Abstract

A sequential study of the invasion of oral mucosa by C. albicans is difficult to accomplish in human beings or in animal models. An in vitro model of oral candidal infection has therefore been established and evaluated by light and electron microscopy.

In the model system cultured oral mucosa from neonatal rats and rabbits was infected with C. albicans. The pattern of invasion of the tissues was similar to that reported in vivo although invasion tended to be more extensive and progressed to the underlying connective tissues.

The system was evaluated by comparing the invasion of keratinized and non-keratinized epithelium by a number of Candida species; their ability to invade oral mucosa was in accord with their differing pathogenicities, and the extent of invasion of the epithelium by the less pathogenic species was related to its degree of keratinization.

The ultrastructural relationship between the superficial epithelium and the fungi was similar to that seen in human oral candidosis, thus validating the model as one in which to study the invasion of epithelium by C. albicans. In examining the fine structure of the invading fungi changes were observed in the organization of the fungal cell wall during the invasive process. Cytochemical localization of acid phosphatase and phospholipase demonstrated that these enzymes are located on the surface of Candida within the tissues although their role in epithelial cell membrane penetration was not clearly established. It is suggested that both fungal enzymes and mechanical force may facilitate fungal invasion.

The deep candidal invasion seen in culture did not result from changes within the epithelium but probably reflected the lack of systemic factors. An evaluation of the role of immune and non-immune serum factors in the model system indicated that these factors alone may not restrict candidal penetration in vivo.
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CHAPTER I

GENERAL INTRODUCTION
Most human beings, indeed probably all living things carry throughout life a variety of microbial agents which are potentially pathogenic for them. Under normal conditions these endogenous parasites do not manifest their presence but live as commensals in an inoffensive relationship with the host. Such parasitism does not evoke an antagonistic reaction in its host and is a sound biologic habitat. However if certain factors upset the balance between man and his endogenous flora the pathogenic nature of these parasites is expressed as the symptoms or lesions of microbial disease. When the causative organisms take advantage of the host they are referred to as opportunistic invaders. Thus infection with such parasites could be said to be the normal state, only the disease response of the host being abnormal in terms of biological co-existence.

Mycotic diseases are significant members of the group of opportunistic infections, and candidosis*, aspergillosis and cryptococcosis are infections which present a world-wide problem magnified in recent years by the mass consumption of antibiotic drugs, the use of immunosuppressive therapy and other technical and therapeutic advances which prolong life expectancy at the cost of host resistance. *Candida albicans*, the principal pathogenic member of the genus *Candida*, (Hurley, 1966) is the perfect example of an organism which has an opportunistic relationship with both man and animals. As a commensal it can be isolated from about 40% of the human population (Barlow & Chattaway, 1969) but within recent years expression of its more aggressive character has occurred with increasing frequency. The ensuing conditions may be localized, as in oral or skin infections or may present as a more severe form of disseminated infection

*The terms Candidosis and Candidiasis are both used in the literature in reference to candidal infections. A discussion at the Symposium on Candidal Infections (1966) upheld the use of the term Candidosis. It is intended to use this term in the present account.
which may prove fatal to the severely debilitated patient. The oral mucosa is one of the most frequent sites of both acute and chronic candidal infection. The acute form is often seen in neonates, and in adults after antibiotic therapy, while the chronic stomatitis is most often associated with wearing of acrylic dentures. Both manifestations prove to be persistent oral problems.

The relationship established between a fungal parasite and man is both interesting and complex for the factors which influence the relationship in superficial infections probably differ from those which play a role in dissemination of the fungus. However the relationship between the micro-organism and the macro-organism is fundamentally one established between cells regardless of whether the host cells are those of the superficial epithelial layers or of the various internal organs which the fungus colonizes. In contrast to the majority of bacterial infections where the invading organisms remain outside the host cells, infection by *C. albicans* has been shown to involve intracellular penetration by the parasites (Montes & Wilborn, 1968). There are other microbial infections such as those involving trypanosomes and viruses in which intracellular parasitism occurs but on the whole these are more aggressive parasites than Candida, depending upon the environment of the host cell for their survival and ultimately bringing about its destruction. The status of the intracellular host-parasite relationship in candidosis is less obvious, for while Stanley and Hurley (1967) reported cytopathic effects of *C. albicans* on renal epithelial cells in culture, observations of invasion of superficial oral epithelial cells in vivo do not suggest significant deleterious effects on the host cells (Cawson & Rajasingham, 1972).

One of the most pertinent questions concerning the intracellular existence of the fungal parasites must be: "How does the fungus invade the host cell?" Studies of fungal parasites of plant and animal tissues have shown that many may be capable of decomposing high molecular weight organic compounds by synthesising hydrolytic enzymes at the fungal cell surface which may diffuse into the surrounding environment (Waid, 1968).
It has also been shown that fungal parasites such as the dermatophytes can exert mechanical force to physically penetrate tissue barriers, although it is probable that such penetration is facilitated by the action of exogenous fungal enzymes (English, 1963). It would seem likely that similar processes operate in the superficial invasion by C. albicans, but there is little evidence available to support these assumptions.

The local and systemic response of the host to an intracellular parasite might be expected to differ from that elicited by extracellular invaders, and in superficial candidal infections this response would seem to be a complex phenomenon. The role of localised host factors, such as the reaction of individual cells to fungal penetration and intracellular growth has yet to be clarified. Similarly the manner in which the systemic response, manifest as an inflammatory reaction in the underlying tissues, is mediated remains a matter for speculation. The presence of an inflammatory reaction in tissues generally results in a disturbance in the turnover of the overlying epithelium, which in oral candidosis is expressed either as an atrophic or hyperplastic response (Lehner, 1966). Chronic candidal infection will give rise to long standing alterations in epithelial maintenance (Cawson & Lehner, 1968; Cawson, 1969), and Candida is sometimes found associated with areas of hyperplastic oral epithelium that histologically show signs of epithelial atypia (Roed-Petersen et al., 1970; Renstrup, 1970). Whether the presence of Candida in these conditions indicates a causal relationship (Cawson & Lehner, 1968) or whether the fungi are merely secondary invaders (Jepsen & Winther, 1965; Shear, 1972) has not yet been decided, although there have been several reports of chronic candidal infections associated with leukoplakia, which have undergone malignant change (Cawson, 1969; Williamson, 1969; Degos et al., 1970; Eyre & Nally, 1971). The possibility that hyperplastic candidal lesions may have a tendency to undergo neoplastic change underlines the importance of a clearer understanding of the pathogenesis of superficial candidosis. The following chapter will review the present knowledge of the fungal infection and the factors involved in its initiation.
CHAPTER 2

A REVIEW OF THE LITERATURE

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2.3 THE HOST TISSUE

2.4 THE LESION
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  2.4.2 Ultrastructural studies of oral candidosis

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  2.5.2 Physiological and hormonal changes
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  2.5.4 Drugs and therapeutic agents

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2.1 INTRODUCTION

The literature relating to the infection of man and animals by fungi of the genus Candida is voluminous, and is said to account for 20% of the medical and veterinary mycological literature today (Ainsworth, 1968). There are several reviews of the historical aspects of the disease (Winner & Hurley, 1964; Higgs, 1973(a)) and according to these, Hippocrates can probably be attributed with the earliest recorded description of an oral candidal infection. Many centuries later candidosis, under the name of 'thrush' was a well known malady of 17th century Europe. The extent of the clinical knowledge of both local and disseminated candidal infections at that time was impressive. In 1771 the Swedish physician Rosen von Rosensein described the progressive dissemination of oral candidosis to involve the lungs and intestine, and recognised the potentially lethal nature of the disease (cited in Higgs, 1973(a)). The association between a fungus and the diseased tissue was observed microscopically in the 17th century but the definition of Candida as the aetiological agent took some years to gain acceptance even after Berg (1866) in his treatise. 'Thrash in children' had argued conclusively that the causative agent was a fungus (cited in Winner & Hurley, 1964).

In the following years the history of medical mycology merged with that of medical microbiology and investigations into the pathogenic fungi were subordinated to those concerned with the aggressively infectious bacterial organisms which so readily plagued man. The discovery of antibiotics while giving rise to the hope that man could be rid of his pathogens, if not all of his parasites in reality brought about a co-incident increase in infections from the less pathogenic organisms unaffected by these therapeutic agents. Commensals such as C.albicans were given the chance to take advantage of the debilitated host and to invade and cause disease and with the increase in the use of antibiotics and similar therapeutic agents in the past three decades the importance of these
essentially weak pathogens has once again become apparent. Investigations
during the last 20 years have not only concentrated on the pathogenic
mechanisms of the fungi but also on the role of host resistance in the
pathogenesis of the disease, and many studies have attempted to define
the variety of changes occurring in the relationship between the host
and the fungus which can lead to the expression of disease in man.

In reviewing the literature related to a study of oral candidosis
the two components involved in the relationship namely, the parasite
C. albicans, and the host tissue must first be discussed. Then, in con-
sidering the nature of the disease, those factors which govern the
host-parasite relationship established in oral candidosis will be
examined. While much of the literature reviewed will be related to
mucosal candidosis, and that of the oral mucosa in particular, it seems
obvious that many common factors must exist between the disease processes
occurring in all forms of superficial muco-cutaneous candidosis, so
that the literature relating to cutaneous candidal infection is also
important. The factors involved in the dissemination of the fungus and
its relationship with the internal systems of the body may differ some-
what from those associated with superficial infections but, nevertheless
the literature dealing with these conditions must be evaluated in order
to obtain information on the balance between host and parasite and the
influence of systemic factors upon local disease.

Before dealing with the components involved in candidosis it is
appropriate to define the disease and set out some of its clinical mani-
festations.

'Candidosis is an acute, superficial or disseminated mycosis
caused by species of Candida. Its clinical varieties are so diverse that
a more specific general definition cannot be given.' (Medical Mycology,
1970).

The above definition, from a recent textbook of medical mycology,
while omitting the chronic infections and allergic reactions which can be
caused by C. albicans, does indicate the considerable spectrum of clinical manifestations of candidosis, some of which are set out in Table 2.1

Table 2.1
Clinical manifestations of candidosis (after Baere et al., 1972)

A. Infective
   (i) Mucosal candidosis including oral, vaginal, bronchial oesophageal and enteric infections.
   (ii) Cutaneous candidosis including intertrigo, onychitis and chronic candidal granuloma.
   (iii) Systemic candidosis including urinary, pulmonary, endocardial, meningeal and septicaemic infections.

B. Allergic including asthmatic and eczematous disease.

Several classifications of oral candidosis appear in the literature. Lehner's (1966) classification divided the disease into acute infections, including candidal leukoplakia and denture associated stomatitis. Higgs and Wells (1973, 1974) based their classification of muco-cutaneous candidosis on aetiological factors, while Walker (1975) has combined these two classifications as shown in Table 2.2. Many of the factors shown in this table which play a role in the aetiology of the disease will be discussed later in the review, but it should be emphasised that many of the manifestations are rare, and only thrush, denture associated stomatitis and possibly candidal leukoplakia occur commonly in the mouth.

ERRATUM
p.17 Paragraph 2
'Lehner's (1966) classification divided the disease into acute infections including candidal leukoplakia' should read
 Acute infections including thrush and antibiotic sore tongue and chronic infections including candidal leukoplakia and denture associated stomatitis...
TABLE 2.2 Classification of oral candidosis (after Walker, 1975)

GROUP I
Oral candidosis as part of muco-cutaneous candidosis occurring in patients with a profound immune deficiency syndrome.

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Inheritance</th>
<th>Distribution</th>
<th>Onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swiss type agammaglobulinaemia</td>
<td>Congenital</td>
<td>Skin, oral</td>
<td>Childhood</td>
</tr>
<tr>
<td>Hereditary thymic dysplasia</td>
<td>or</td>
<td>mucosa,</td>
<td></td>
</tr>
<tr>
<td>Di George syndrome</td>
<td>genetically</td>
<td>nails</td>
<td></td>
</tr>
<tr>
<td>Chronic granulomatous disease of childhood</td>
<td>determined</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

GROUP II
Chronic muco-cutaneous candidosis remains superficial. In subgroup 4 only the mouth is usually involved. All patients have chronic hyperplastic oral candidosis.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Genetic group</th>
<th>Age onset</th>
<th>Type</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Autosomal recessive</td>
<td>&lt; 10 yrs.</td>
<td>Familial chronic muco-cutaneous candidosis</td>
<td>Mouth, nails, skin. Other sites sometimes affected</td>
</tr>
<tr>
<td>2</td>
<td>Unknown</td>
<td>&lt; 5 yrs.</td>
<td>Diffuse chronic muco-cutaneous candidosis</td>
<td>Mouth, skin and nails extensively involved. Eyes, pharynx and larynx</td>
</tr>
<tr>
<td>3</td>
<td>Autosomal recessive</td>
<td>By second decade</td>
<td>Candida — endocrinopathy</td>
<td>Mouth, hypoparathyroidism, hypoadrenocorticism, hypothyroidism &amp; diabetes mellitus</td>
</tr>
<tr>
<td>4</td>
<td>Probably not genetically determined</td>
<td>&gt; 30 yrs.</td>
<td>Late onset</td>
<td>Chronic hyperplastic oral candidosis</td>
</tr>
</tbody>
</table>

GROUP III
This group includes common forms of oral candidosis, usually confined to the mouth. The candidosis is usually transient (1) and (2), easily treated and often a local or general predisposing factor is present; it may occur at any age. There is no recognised hereditary predisposition.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Clinical features</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Acute psseudomembranous candidosis (thrush)</td>
</tr>
<tr>
<td>2</td>
<td>Atrophic candidosis</td>
</tr>
<tr>
<td>3</td>
<td>Chronic atrophic candidosis</td>
</tr>
</tbody>
</table>
2.2 THE FUNGUS

Candida is a genus of the subfamily Cryptococcoidea of the asporogenous yeasts. Such yeasts were described by Skinner (1947) as 'true' fungi whose usual or dominant form is unicellular. However the exceptional characteristic of C. albicans and closely related species is that besides existing as the unicellular yeast or blastospore (the "Y-phase"), it also exists in a mycelial or vegetative form (the "M-phase"). The literature contains conflicting opinions as to whether this mycelial phase represents a true mycelium or a pseudomycelium. True mycelia are formed by the laying down of cross walls along the tubular outgrowth of the parent blastospore although physiological continuity along the entire length of the hypha is maintained. Pseudomycelia on the other hand arise from the approximation of a number of separate elongated cells end to end through which there is no functional continuity (Lodder, 1934 reported in Winner & Hurley, 1964). While early opinion favoured the formation of pseudohyphae by C. albicans (Scherr & Weaver, 1953) Gresham and Whittle's (1957) biochemical studies are cited as evidence that C. albicans produces a true mycelium (Seelig, 1966). However as it is difficult to determine conclusively whether the true hyphal or pseudohyphal form is present and either term seems permissible in the literature, the vegetative form will be referred to as the mycelial or "M-phase" in this thesis. C. albicans also exists in a third form-the clamydospore; these thick-walled structures usually develop in environments which lack nutrients although they have been reported in human and animal candidosis (Heinman et al., 1961; Winner & Hurley, 1964). It has been suggested that their function is merely that of a storage cell (Bakerspigel & Burke, 1974).

The change from any one form of Candida to another is totally reversible in true strains of C. albicans and many factors have been found to promote the growth of one form over another. Talice (1930 cited Winner & Hurley, 1964) postulated that favourable environmental conditions encouraged the growth of the yeast phase whereas in adverse
conditions the M-phase predominated. Such a simplistic view was contested by McClary (1952) who showed optimal growth of either morphological form needed certain specific conditions. Since that time the literature has abounded with reports of the conditions and environmental factors which promote filamentation of *C. albicans in vitro*. Differences in both the ultrastructure (Cassone et al., 1973) and biochemical composition (Chattaway et al., 1968) of the cell walls of the yeast and mycelial forms have now been demonstrated which emphasises that definite metabolic changes occur during the transformation. Interest in the determinants of the yeast-hyphal transformation stems from the possibility that only one form is the pathogenic phase, capable of tissue invasion. However, there is much conflicting evidence about the pathogenicity of the two forms, and conclusions have usually been based upon the type of organism which predominated in smears and sections of infected tissues where, although both Y-phase and M-phase are often present, the mycelial forms generally predominate. The early literature on this subject has been amply reviewed by Winner and Hurley (1964, 1966) and will not be considered here.

More recently Hurley and Stanley (1969) have showed an association between the production of the M-phase by *Candida* species and fungal invasion and cytopathic effects on cultured epithelial cells, although they had earlier claimed that no qualitative difference in the cytopathic effects of the M and Y-phase of *C. albicans* could be observed and they suggested that the Y-phase may initiate infection in vivo (1967). Considerable confusion still exists in the literature; Mardon et al. (1975) confirmed earlier work by Simonetti and Strippoli (1973) which showed that the Y-phase had a greater lethal effect than the M-phase when inoculated intravenously into mice. On the other hand Saltarelli et al. (1975) found mycelial-forming strains more virulent. However these workers were testing morphologically mutant strains of *C. albicans*, the virulence of which may not be related to normal strains. One hypothesis offered by those who favour the Y-phase as the predominant pathogenic form is that the development of the "less aggressive" M-phase in vivo is
encouraged by host defence mechanisms, as a way of restraining the "more pathogenic" Y-phase (Mardon et al., 1975). The interrelationship between form and pathogenicity remains an intriguing and complex problem and may very well depend on the type of tissue with which the fungus in interacting, or indeed the strain or sex of the animal (Saltarelli et al., 1975).

While the pathogenicity of the two forms remains a matter of dispute, controversy also centers upon the nature of the mechanism underlying the yeast to mycelial transformation in vivo. A variety of body fluids and exudate including serum, saliva and cell exudates have been found to support the formation of germ-tubes, the initial stage in the development of the M-phase, from blastospores of C. albicans in vitro. Barlow et al. (1974) have isolated protein-associated factors present in both serum and seminal plasma which promote development of the M-phase in the presence of glucose at 37°C. and other serum factors, such as transferrin (Caroline et al., 1964; Easterly et al., 1967) and specific anti-candidal antibodies (Grappel & Calderone, 1976), have also been claimed to affect the morphological form and viability of C. albicans. The effect of body fluids in vivo is a complex problem which is more relevant to discussion of host defence mechanisms and so will be considered in section 2.7.

As well as the partially defined serum factors which influence the fungal form of C. albicans there are factors present within, or associated with, the epithelial cells which may affect form determination. One hypothesis which has gained support in recent years is that the intraepithelial micro-environment, and in particular the concentration of constituent amino-acids such as cysteine and cystine, influences form. Taschdjian et al. (1960) demonstrated the susceptibility of mouse vagina to candidal infection during oestrus when high disulphide levels are present in the vaginal epithelium. These workers showed that the Y-phase predominated in the fully keratinized layers, which presumably contain high levels of cystine while the M-phase was present in the granular layer where high concentrations of cysteine may exist. This lead to the hypothesis that form determination may be in-
fluenced by external changes in the disulphide sulphydryl equilibrium (Higgs, 1973(b)). Clinical studies on patients suffering from chronic muco-cutaneous conditions led Higgs (1973(b)) to suggest that differences in the amount of sulphur-containing amino-acids in the epithelia of different subjects might affect the individuals susceptibility to candidal infection. However Wain et al. (1975), in a biochemical assessment of the effect of cysteine on the growth of C. albicans in vitro, were not able to support the concept of cysteine-mediated form determination.

While the gross morphology of C. albicans has aroused a considerable amount of interest, studies of the fine structure of the various forms of the fungus are less extensive. Mycologists have investigated the ultrastructure of the readily cultivated yeast forms of Candida for some years, but it is only recently that studies of germ-tube production and the mycelial form have been carried out. Early studies of the blastospores of the genus Candida by such workers as Iwata and Hirata (1963, 1966), Bakerspigel (1964), Tsukahara and Sato (1964), Montes et al. (1965) indicated the presence of organelles common to most eukaryotic cells, contained within a plasma membrane and outer cell wall. However in many of these studies inadequate fixation led to poor resolution of subcellular structures. Recently Borgers and DeNollin (1974) have overcome these problems and obtained excellent electron micrographs of intracellular components. These workers showed the yeast cell to contain an irregularly shaped nucleus bounded by a membrane and surrounded by cytoplasm, whose density depended upon the number of ribosomes present. Mitochondria were distributed throughout the cytoplasm and a vacuolar apparatus was present consisting of amorphous, electron-lucent material bounded by smooth membrane. The presence of other fragments of smooth membranes, vesicles and lipid bodies seemed to depend on the growth phase of the organism. The demonstration of the nature and possible functions of such organelles has been attempted by enzyme cytochemistry, but the distribution of specific enzymes which act as organelle markers differed from that in mammalian cells (DeNollin et al, 1975). The characteristic fungal cell wall of Candida has been the subject of
particular interest because of its probable association with antigenic determinants and other factors such as extracellular enzymes, which may contribute to the pathogenicity of the fungus. The cell wall consists of a multilayered structure with amorphous granular and fibrous components of differing electron-density (Djaczenko & Cassone, 1972). Borgers and DeNollin (1974) described three distinct layers while Djaczenko and Cassone (1972) were able to demonstrate five layers, including the invaginated plasma membrane which lies on the inside of the cell wall. Later studies showed modifications in this structural pattern which occur in germ-tube formation (Cassone et al., 1973). The only accounts of the fine structure of the mycelial phase of C. albicans have arisen from studies on human tissues infected with the fungus (Montes & Wilborn, 1968; Cawson & Rajasingham, 1972; Cawson & Rajasingham, 1973). These showed the mycelial phase to have a similar ultrastructural organization to the yeast form.

2.3 THE HOST TISSUE

Skin and mucosa constitute the anatomical barrier which, by covering or lining the body surfaces, protects the organism from its environment; in superficial candidosis it is these tissues which become the habitat of C. albicans. Both skin and mucosa consist of a stratified epithelium supported by a loose connective tissue. The surface cells are functionally adapted to form a protective layer and are continually replaced by differentiation of cells that arise from division in the basal layer. Imposed upon this basic pattern is a specialisation of the epithelial organization which meets the specific functional requirements of the different regions of the skin and mucosa.

The mucosa which lines the oral cavity is a moist lining tissue in which epithelial structure ranges from an orthokeratinized epithelium similar to epidermis to non-keratinized epithelium. However, oral keratinization is not as simple as this description would suggest, for different degrees of keratinization are seen in the mucosa of most
mammals and a better representation of keratinization is that of a
spectrum varying in small steps between the extremes of full ortho-

The least differentiated cells in the oral epithelium consist of
cuboidal or columnar cells in contact with the basement membrane and
forming the basal layer. Above these are the large cells of the prickle
cell layer, which, in keratinizing epithelia, differentiates to form
more flattened cells containing keratohyalin granules which constitute
the granular layer. Cells in all these layers are nucleated. The most
superficial cell layer of keratinized tissues consists of flattened non-
nucleated cells or squames packed with the filamentous protein, keratin.
This consists largely of insoluble fibrous protein with a high proportion
of the sulphur-containing amino acid cystine; these proteins differ widely
from region to region, and from species to species, but are usually in-
cluded under the generic heading of keratin (Iqbal & Gerson, 1971).
Ultrastructural studies (Brody, 1959) show keratin to consist of aggre-
gates of fine fibrils essentially similar to the tonofilaments seen in
the deeper cells of the epithelium, but embedded in an electron dense
matrix. The cell membrane of the squame is thickened and extremely
resistant to most chemical agents (Matoltsy & Parakkal, 1967). In
tissues which are parakeratinized it seems likely that formation of
keratin proceeds in a normal way but is not accompanied by the removal
of all organelles and in particular the nuclei, which thus persist in
surface cells. Variants of mammalian oral epithelium show not only
partial loss of organelles, but different degrees of packing of tono-
fibrils and in some cases no matrix formation (Alvares & Meyer, 1971).
In the extreme variant, non-keratinized epithelium, such as is seen
for example in human buccal epithelium, there is neither packing of tono-
filaments nor matrix formation and most of the cell organelles including
nuclei remain in the surface cells (Silverman, 1971).

Besides functioning as a protective mechanical barrier, oral epi-
thelium also serves as a permeability barrier to the passage of
substances across the tissue (Squier & Johnson, 1975); this barrier is
apparently located in the superficial level in both keratinized and non-keratinized regions of the oral mucosa (Squier, 1973; Squier & Rooney, 1977).

Although any of the tissues described above may act as host to the fungi in superficial candidosis, the possibility that the type of epithelium present might influence the extent of candidal invasion has not been fully investigated. Colonization of the tissue surface is obviously an important factor and, depends on both the type of tissue and its physiological state. Normal dry skin with its fungicidal exudate is an efficient defence barrier against Candida unless the tissue becomes macerated, when fungal invasion may ensue. Maibach and Kligman (1962) found that an occlusive dressing was necessary before experimental infection of the skin could be produced and merely damaging the skin did not allow infection to take place. However in the same study these workers were unable to produce experimental infection of the oral mucosa even when the inoculum was occluded with Orabase. This probably reflects the different properties of the oral epithelium which, unlike the skin retains its structural integrity when moist.

The first experimental evidence that the type of epithelial keratinization may influence fungal invasion came from Taschdjian et al. (1960) who suggested that C. albicans has a preference for fully orthokeratinized epithelium and have showed that in mouse vagina infection only took place during the period when the vaginal epithelium was fully keratinized although other environmental factors, such as the change in pH of the region, may have promoted infection (see section 2.5.2). Infection of the various epithelial surfaces of the oral mucosa in humans seems to occur more often than infection of orthokeratinized skin, but this may merely reflect the different carriage rate of C. albicans in these regions (see Table 2.4, section 2.6.1) for as previously mentioned experimental infection of skin was achieved more easily than of buccal mucosa (Maibach & Kligman, 1962).

On the whole, the literature contributed little information on the relative frequency of candidosis in different regions of the mouth of
Humans or animals; candidal infections would not seem to have any clear regional predilection except in denture-associated stomatitis which is restricted to denture-bearing mucosa. The factors governing the extent and characteristics of the area infected by the fungus are complex and few firm conclusions can be drawn.

2.4 THE LESION

2.4.1 The histopathology of oral candidosis

Cawson (1966, 1976) has given several detailed accounts of the histopathology of oral candidosis dividing the oral infection into two basic types on histopathological grounds, the acute form represented by thrush and the chronic form by candidal leukoplakia. In both acute and chronic lesions the candidal infection is seen as a hyphal penetration of the superficial epithelial cell layers and the host response as an inflammatory lesion. The duration of the infection governs the type of inflammatory infiltrate which in turn influences the response of the epithelium so that a spectrum of histopathological changes is seen.

Thrush, clinically an acute florid infection appears histologically as an oedematous plaque on the surface of the epithelium formed by desquamating nucleated epithelial cells matted together by hyphae and spores of C. albicans. The inflammatory infiltrate consists predominantly of polymorphs and spreads throughout the epithelium, the main localization occurring between the spinous layer and the para-keratinized layers. In candidal leukoplakia the numbers of invading organisms may be less but the response of the underlying tissue is often equally intense; hyphae penetrate the epithelium only as far as the spinous layer. The epithelium, whether originally keratinized or non-keratinized, generally becomes para-keratinized, and some degree of epithelial hyperplasia is always present. Frequently the hyperplasia is seen to be severe (Cawson & Lehner, 1968; Cawson, 1969) while there are a number of reports of epithelial dysplasia occurring in these lesions (see section 1). The inflammatory reaction spreads throughout the epithelium and connective
tissue and while polymorphs are generally present superficially, the deeper inflammatory infiltrate consists predominently of chronic inflammatory cells such as lymphocytes and plasma cells.

2.4.2 Ultrastructural studies of oral candidosis

Studies on the fine structure of *C. albicans* alone have already been mentioned (section 2.2); fewer investigations of the ultrastructural relationship between *C. albicans* and host tissues in candidosis are to be found. Montes and Wilborn (1968) studied the host-parasite relationship in superficial candidosis. They examined superficial epithelial squames of tongue and buccal mucosa from patients suffering from candidal infection, and made the unexpected and important finding that *C. albicans* is an intracellular parasite which penetrates the cytoplasm of the epithelial cells. Breakdown of the epithelial cell plasma membranes at the site of fungal entry and the loss of intracytoplasmic tonofibrils adjacent to the intracellular fungi was also reported. Montes and Wilborn suggested that this was due to fungal enzyme activity. Cawson and Rajasingham (1972 & 1973) provided a more detailed account of the fine structure of the fungus and its relationship with cells of the oral epithelium. In contrast to the findings of Montes and Wilborn, Cawson and Rajasingham found that there was virtually no disruption of the organization of the host cell by fungi penetrating the cell membrane or present within the cell cytoplasm. The epithelial cell membranes appeared intact and no loss of cytoplasm or tonofibrils around the invading hyphae was seen. The recent work of Mohammed (1975) has once again raised the possibility that cytoplasmic degeneration of the epithelial cells may be associated with intracellular growth of fungi, although his interpretation is that such degeneration is associated with the long term presence of fungi rather than with their recent invasion. Montes and Wilborn and Cawson and Rajasingham used mainly surface smears of candida lesions and could not demonstrate the effects of *Candida* on the epithelium as a whole. However Mohammed (1975) took biopsies from a
patient with chronic candidal leukoplakia, and reported that the deeper epithelial cells showed signs of increased metabolic activity, containing numerous mitochondria and ribosomes. Other studies of the ultrastructural relationship established between Candida and other tissues have been reported. For example Schnele and Voigt (1974) used biopsies from vaginal candidal lesions but their studies did not contribute any further information about the epithelial-fungal relationship in vivo.

2.5 PREDISPOSING CONDITIONS FOR CANDIDAL INFECTION

The alterations in the status of the host which increase the incidence of candidosis are of interest in view of the light they shed on the mechanisms involved in candidal infection. Candida infections arise when conditions are favourable for proliferation of the fungi within the host, such as during antibiotic therapy or in hyperglycaemic patients, or because the host defense mechanisms are depressed as during immunosuppressent or radiation therapy. Systemic and local predisposing factors for candidal infections have been extensively reviewed in literature (Budtz-Jorgensen, 1974; Selliger, 1975; Walker, 1975). Table 2.3 illustrates the wide range of factors which have been recognized as predisposing man to candidal infections. The mechanism by which these factors operate is unclear and the following discussion will concentrate on what is known of such mechanisms with regard to superficial candidosis.

<table>
<thead>
<tr>
<th>Table 2.3 Factors predisposing to infection by C. albicans (after Winner, (1969))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Physiologic:</td>
</tr>
<tr>
<td>Pregnancy</td>
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<tr>
<td>Early infancy</td>
</tr>
<tr>
<td>2) Traumatic</td>
</tr>
<tr>
<td>Maceration of skin</td>
</tr>
<tr>
<td>Chemical damage to mucous membrane</td>
</tr>
<tr>
<td>3) Allergic conditions of the skin</td>
</tr>
<tr>
<td>4) Endocrine disorders:</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
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<tr>
<td>Hypoth_yroidism</td>
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<tr>
<td>Hypoparathyroidism</td>
</tr>
<tr>
<td>Hypoadrenalism</td>
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<tr>
<td>5) Malnutrition and malabsorption syndrome</td>
</tr>
</tbody>
</table>
6) Malignant diseases, especially leukaemias
7) Agranulocytosis and aplastic anemia
8) Postoperative states
9) Antibacterial antibiotic therapy
10) Immunosuppressive drug therapy

Basically, a predisposition to mucosal or skin candidosis would seem to be mediated through one of two pathways; either a local change alters the status of the epithelium, or an inadequate systemic response allows an infection to persist. In some situations the predisposing factor may result in both outcomes as in nutritional deficiencies where local epithelial dystrophy and impairment of the immune response together may facilitate infection. In such cases it is often difficult to tell which factor predominated in the aetiology of the disease.

2.5.1 Congenital abnormalities

Chronic mucocutaneous candidosis has been observed in several patients suffering from inherited disorders of keratinization of oral mucosa, skin and nails (Wells et al., 1972). Kirkpatrick et al. (1971) found 11 out of 12 patients with chronic mucocutaneous candidosis to have dysplastic teeth which Walker (1975) suggested might indicate an ectodermal defect that predisposes to candidosis.

2.5.2 Physiological and hormonal changes

Naturally occurring physiological changes in the status of an epithelium are often hormonally mediated as for example, those seen in pregnancy, infancy and old age. The most striking example of an epithelial change predisposing to fungal infection is that of the vagina during pregnancy. Selliger (1975) suggested that the increased glycogen content of the epithelial cells might increase colonization by fungi, but evidence stemming from experimental infection of mouse vagina argues against such direct effects (Taschdjian et al., 1960) and has suggested that the increased incidence of candidosis is due to extraepithelial
changes in the environment of the vagina such as the presence of bacteria and shifts in pH which promote fungal growth. In fact the unusually low pH present in neonates mouth and the vagina during pregnancy has been considered significant in the etiology of acute candidosis of these regions (Beare et al., 1972).

The predisposition of the diabetic patient to candidosis is an interesting one. In general such patients have a reduced resistance to infection and one theory suggests that it is hyperglycaemia which promotes growth of Candida (Barlow et al., 1974). However there is conflicting evidence regarding colonization of the oral cavity where an increase has been reported in the diabetic by Basu et al. (1961) while a study by Peters et al. (1966) showed no such predisposition to colonization in diabetics, controlled or otherwise. Russell and Jones (1973(c)) studying experimental infection of rat mucosa in vitro showed that a carbohydrate diet increased carriage of Candida and possible infection by the fungus, while Knight and Fletcher (1971) demonstrated that glucose in saliva encourages the growth of C. albicans. Thus it seems likely that increased numbers of Candida may be carried by diabetic patients and that if diabetes is not well controlled, the resistance of the host will be reduced sufficiently to allow infection to take place.

Besides diabetes there are a number of reported instances of patients with other hormone deficiencies mentioned in Table 2.3 such as hypoadrenalism, hypoparathyroidism who show increased candidosis. What is interesting is that in some of these infection with Candida precedes the manifestation of the endocrine disorder. This has lead to the proposal that candidosis is able to cause endocrine disturbances, but little supportive evidence has been presented (Cawson, 1976). The many changes that have been reported in the properties of membranes and distribution of electrolytes and metabolites as a result of hormone imbalance led Barlow (1969) to put forward the view that it is a combination of such changes that render the cell more susceptible to attack by C. albicans so creating both increased incidence of disease and an increased number of carriers.
2.5.3 Nutritional deficiencies

There are few definitive studies on increased susceptibility to candidal infection due to malnutrition or malabsorption of essential nutrients. However, a number of findings would tend to suggest that deficiencies of such substances as iron, Vitamin A, and pyridoxine may lead to dystrophic changes of the epithelium with a resultant increased susceptibility to candidosis (Higgs & Wells, 1972; Wells et al., 1972). Once again the difficulty is in defining epithelial dystrophy as the aetiological agent when malnutrition must also have an effect on host defense mechanisms.

Iron deficiency is thought to be an important predisposing factor in superficial candidosis. Either frank anaemia or latent iron deficiency is found in 70% of patients with chronic muco-cutaneous candidosis (Higgs and Wells, 1972). Iron deficiency in candidosis with a later onset, such as denture associated stomatitis and candidal leukoplakia is rarer. However, Bothwell and Finch (1962) and Rose (1968) related glossitis and angular cheilitis to low levels of plasma iron. Walker et al. (1972) in a controlled study showed no difference in the frequency of candidosis between populations which were and were not iron deficient. Various explanations have been proposed for the mechanism by which the incidence of candidosis is increased in iron deficiency. Fletcher et al. (1975) have shown that salivary counts of oral flora including C. albicans, increase when low levels of iron are present so the likelihood of colonization and ensuing infection may well increase in such circumstances. Alternatively, Jacobs (1960, 1961(a) & (b)) has claimed that chronic lack of tissue iron can result in "defective" epithelial formation due to a lack of iron-containing enzymes in the epithelial cells; this defective epithelium might allow infection to take place more readily. An alternative concept of the mechanism of iron activity has arisen from the studies of Joynson et al. (1972) who demonstrated an impaired cell mediated immune response to C. albicans in iron deficient subjects, which was restored by iron therapy. This suggests once again that the effects of iron deficiency may be mediated centrally rather than locally via the epithelium.
Wells et al. (1972) and Montes et al. (1973) have related chronic muco-cutaneous candidosis to hypovitaminosis A. Vitamin A is known to affect the keratinization of epithelia, lack of the vitamin promoting keratinization. Montes et al. proposed that such abnormalities may increase epithelial susceptibility to fungal invasion, and an increased incidence of candidosis has been demonstrated in rats deficient in Vitamin A (Lopez, 1946 cited in Montes et al., 1973). However Vitamin A also affects cell membrane stability (Logan, 1972) and it may be a defect at the molecular level which permits invasion of the cells by Candida (Montes et al., 1973).

2.5.4 Drugs and therapeutic agents

While immunosuppressive and cytotoxic drugs might be expected to effect systemic defences against candidal infection, the action of antibiotics and synthetic corticosteroids in promoting candidal infection may be more localised. Seelig (1966) has reviewed the extensive literature relating to the effect of antibiotics on the incidence and severity of candidosis, and concluded that antiobiotics encourage the proliferation of C. albicans in the intestines and on mucosal surfaces. The generally accepted mechanism by which broad spectrum antibiotics promote candidal infection is by the removal of competing bacterial flora, allowing overgrowth by Candida. However, it has also been variously suggested that the antibiotics may facilitate fungal invasion by causing local tissue damage (Seligmann, 1953) and by inhibiting antibody synthesis (Gorczyca & McCarty, 1959) and by inhibiting phagocytic activity (Takahashi et al., 1958) all of which compromise resistance.

While the activity of antibiotics such as penicillin and streptomycin may reside primarily in suppression of competitive flora it is possible that tetracyclines may have specific factors which encourage colonization and invasion by C. albicans. Russell and Jones (1973(a)) demonstrated that experimental infection of rat mucosa was facilitated by tetracycline administration, and the effect was maintained for a considerable time after therapy was discontinued, when the normal oral microbial flora had probably been re-established (Russell et al., 1976).
This would suggest that the drug may modify host tissue reaction rather than merely suppress the oral flora, and recent in vitro studies suggest that tetracyclines may be associated with reduced chemotaxis of leucocytes (Martin et al., 1972).

The mechanism by which corticosteroids predispose to infection with C. albicans is rather uncertain; these agents seem to increase the ease with which the fungus invades the epithelium rather than affecting the growth or the organism per se. Steroids have a diabetogenic effect and increased salivary glucose concentration along with increased blood and tissue glucose have been demonstrated in patients treated with corticosteroids (Knight & Fletcher, 1971) and this in itself would encourage fungal growth. Topical steroids with an anti-inflammatory effect are well known to encourage candidosis. For example beclomethasone diprropionate inhaled in powder form for asthma predisposes many patients to pharyngeal candidosis (Brompton Hosp.--M.R.C., 1974) although oral administration of steroids does not seem to promote candidosis to any great extent (Lehner & Ward, 1970). While toxic damage to the mucosa may occur during topical application, the general mechanism by which cortisone is thought to be effective is by local suppression of immune response. It is also believed that cortisone stabilizes lysosomal enzymes (Weissmann, 1969) and so could limit the destruction of fungal cells by phagocytic processes. In this context it is relevant to note that Louria et al. (1960) demonstrated that cortisone depressed the leucocyte response of mice to C. albicans and so caused increased mortality in experimental infections.

2.6 THE DEVELOPMENT OF THE LESION

The development of endogenous candidosis probably progresses through three phases: colonization, epithelial invasion and, in cases where dissemination occurs, haematogenous spread.
2.6.1 Colonization

As commensals Candida are carried commonly by man and other animals. The most common human carrier sites are the mouth, gastrointestinal tract and vagina. The reported prevalence of carriers varies, probably reflecting changes in populations and in methods of isolating the yeast. Table 2.4 lists representative surveys for the carriage of Candida.

Table 2.4

<table>
<thead>
<tr>
<th>Carriage Rate ( % of population evaluated)</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barlow &amp; Chattaway 1969*</td>
<td>45% all sites</td>
</tr>
<tr>
<td>Stenderup &amp; Pederson 1962</td>
<td>53% mouth</td>
</tr>
<tr>
<td>Bartels &amp; Blechman 1962</td>
<td>33-40% mouth</td>
</tr>
<tr>
<td>Winner &amp; Hurley 1964</td>
<td>25% vagina (pregnant)</td>
</tr>
<tr>
<td></td>
<td>16% vagina (non-pregnant)</td>
</tr>
<tr>
<td></td>
<td>30% faeces</td>
</tr>
</tbody>
</table>

(*Survey of a hospital population)

With so many members of the population carrying the fungus, the transfer of C.albicans from person to person must be constantly occurring and this factor is obviously related to the high incidence of candidosis compared to infections arising from non-commensal fungal pathogens. It is also interesting that despite the continual contamination of the skin from other body surfaces it is not usually possible to culture Candida from the epidermis. Presumably the dry intact skin surface with its fungicidal surface film is not a suitable habitat for Candida.

2.6.2 Epithelial invasion

In the order to overcome the natural barriers of the host C.albicans invades epithelial cells and becomes an intracellular parasite as has been
demonstrated by ultrastructural studies of infected tissues (see section 2.4.2). While *C. albicans* has been shown to invade most epithelia of the body including those of skin and mucosa under the appropriate conditions, it seems to have a predilection for mucosa, which may in part reflect the preferential surface colonization which occurs in such tissues. As the structure of the superficial epithelial cells differs with regard to properties such as keratinization and moistness in various regions of the skin and oral mucosa it might be expected that the ability of *C. albicans* to penetrate and colonize different regions would vary although there is little evidence to support such a view (section 2.3). However while *C. albicans* is capable of invading epithelial cells, the striking feature of the lesion in vivo is the restriction of the fungi to the superficial layers of the epithelium. This is seen in oral mucosa, (Cawson, 1966) vagina (Taschdjian et al., 1960) and skin (Maibach & Kligman, 1962) under both natural and experimental conditions. The penetration of the gastro-intestinal mucosa would seem to be more extensive than in oral mucosa and deep mucosal invasion of oesophageal and stomach epithelia has been reported in severely debilitated patients (Beemer et al., 1954; Parker et al., 1976) and in experimentally infected rats (DeMaria et al., 1976).

The reason why *C. albicans* is generally restricted to the most superficial epithelial layers is an intriguing question which has provoked much discussion over the past years. The original theories centered upon the 'inability' of fungi to invade 'living cells' and Maibach and Kligman (1962) suggested that Candida was similar to the keratophilic fungi (e.g. the dermatophytes) which reside only in the 'dead' cells of a keratinized epithelia. However, systemic infection of laboratory animals (Hurley, 1964) and the experimental infection of viable epithelial cells in tissue culture (Stanley & Hurley, 1976) clearly demonstrated that *C. albicans* was able to invade living tissue. Both the "micro environment" of the epithelial cells (Taschdjian et al., 1960; Higgs et al., 1974) and the presence of systemic host factors such as serum (Blank et al., 1959) have been claimed to prevent deep invasion of
fungi in vivo. Such factors obviously are closely related to host-resistance and will be discussed further in that section of the review.

*C. albicans* is one of the special band of parasites adapted to living in what Moulder (1974) called the 'extreme environment' of the mammalian cell. The mechanism by which the fungi gain access to the environment is perhaps one of the most relevant subjects in a discussion of its pathogenicity, but an understanding of the exact invasive mechanism employed by *C. albicans* is still at an early stage. In the introduction to this thesis the types of mechanisms employed by different types of parasitic fungi to invade host tissue and obtain nutrients were briefly mentioned. There is little evidence to suggest whether enzymatic digestion or mechanical force predominates or even wholly accounts for the penetration of epithelial cells by *C. albicans*. Ultrastructural observations of candidal infected epithelium (see section 2.4.2) show little disruption of host cells associated with penetrating fungi (Cawson & Rajasingham, 1972) so whichever mechanism is operating it would seem to be localized in its action. There have been suggestions that endotoxin produced by large numbers of fungi on the surface of the epithelium may damage the tissue sufficiently to allow entry of fungal cells (Maibach & Kligman, 1962). The invasion of keratinized surfaces by *C. albicans* led Kapica and Blank (1957) to try to demonstrate a keratinase associated with *C. albicans*. The fungus was cultured in vitro with keratin as the sole source of nitrogen, but the considerable time lag before any demonstrable breakdown of keratin occurred diminishes the possibility that such an enzyme can play a role in epithelial invasion in vivo. It must also be remembered that keratin is not the only constituent of superficial epithelial cells and many other substances are present which could act as substrates for *C. albicans*. Biochemical studies have shown that *C. albicans* can elaborate other hydrolytic enzymes such as peptidase, phospholipase and phosphatases, which may well play a role in the pathogenicity of the organism. The most comprehensive study, by Chattaway et al., (1971), reviewed earlier biochemical research in the field and examined both M-phase and Y-phase of *C. albicans* for the presence of specific enzymes. The activities of hydrolytic enzymes were found to
vary in the two forms of the fungi and may reflect differing pathogenicities of the M-phase and Y-phase. However while such biochemical studies demonstrate the presence of enzymes in the fungi in vitro they say nothing about their activity in vivo. Cytochemical studies at the ultrastructural level by DeNollin and Borgers (1975) have clearly demonstrated the location of several hydrolytic enzymes including acid and alkaline phosphatase in the yeast form of \textit{C. albicans}. These enzymes were located intracellularly and showed little association with the cell wall which would have been expected if they were to exert their effect at the cell surface. A hydrolytic enzyme which could be significant in facilitating the penetration of cell membranes is phospholipase. Although Chattaway et al. (1971) failed to identify the enzyme by biochemical assay it has recently been demonstrated cytochemically in yeast cells of \textit{C. albicans} in vitro (Pugh & Cawson, 1975).

2.6.3 Dissemination

The evidence would tend to suggest that processes leading to local tissue invasion by \textit{C. albicans} are not necessarily the same as those leading to dissemination of the fungus. Krausse et al. (1968) have shown \textit{C. albicans} to be capable of passing through the normal bowel wall of healthy subjects and this, together with the introduction of fungi into the blood stream via such routes as intravenous catheters and injections, may well act as a path of infection. This is not to say that localized superficial candidosis cannot develop into a progressive septicaemia for once through the superficial barrier it seems \textit{C. albicans} is capable of colonizing blood vessels and then the deep parenchymal organs and Kozinn (1969) has commented on the frequency of mucosal invasion in cases of systemic candidosis. In a study of patients with such lesions Parker et al. (1976) found involvement of the kidney in 80% of the cases, of the brain in 54% and of the heart in 32% although any tissue seemed susceptible. While extensive colonisation of organs must cause considerable mechanical damage the mechanism of tissue invasion and subsequent disruption has always been considered to result
from endotoxin released during proliferation and after death of *C. albicans*. The function of toxins was first indicated by the variety of deleterious effects induced in mice, rabbits, dogs and humans after injection of extracts of fungi or culture filtrates and the early literature has been amply reviewed by Seelig (1966) and by Chattaway et al. (1971). The latter workers however have failed to reproduce many of these earlier findings, and they suggest that toxin activity is dependent upon strain characteristics rather than culture conditions. A range of pathogenicity or virulence among different strains of *C. albicans* has been demonstrated in animals (Mourad & Friedman, 1961) and in chick embryo (Wain & Cawson, 1976) and chorio-allantoic membrane (Partridge et al., 1971) but little substantive proof has been offered that strains differ in their ability to cause disease in man.

Many of the recent investigations into the production of toxins by *Candida* have attempted to relate fungal endotoxin to its bacterial counterpart and both pyrogenic substances (Cutler et al., 1972) and toxic glycoproteins (Nosal et al., 1974) have been identified. These fungal preparations exerted toxic effects similar to bacterial endotoxin but only at much higher concentrations (Cutler et al., 1972), and it is uncertain whether they are normally present in sufficient quantities to play an active role in pathogenicity. However Kind et al. (1972) have shown that mannan extracted from *C. albicans* is able to induce anaphylactic type reactions in mice, probably by activating complement in a similar manner to bacterial endotoxin and at levels which could be encountered in vivo. Alternatively Chattaway et al. (1971) suggested that pathogenicity may be related more to the action of enzymes elaborated by the micro-organism during its growth, rather than the widespread effects of "toxins". Recently however Wain and Cawson (1976) found evidence of an endotoxin-like activity associated with candidal infection of chick embryo which depressed the immunological competence of the embryo.

One remaining feature which contributed to the pathogenicity of the invading organism is its ability to avoid stimulating or to counter-attack the host's defence mechanisms. Relatively little host response
has been observed where *C. albicans* is present either as a superficial parasite in man (Winner, 1969) or in disseminated infection in animals, where heavy colonization of organs occurs (Winner & Hurley, 1964). The perfect parasite is one which does minimal damage to its host and so induces little host response. If host response can be evaluated in terms of inflammatory reaction, then *C. albicans* does seem to behave in this way.

### 2.7 HOST DEFENCE MECHANISMS IN SUPERFICIAL CANDIDOSIS

In this section some of the host defence mechanisms which may be active in oral candidosis are discussed. Essentially there are two lines of defence against the parasite, the first is represented by the environment of the oral cavity and the epithelial barrier while the second is found in the various local and systemic factors associated with the mucosa.

#### 2.7.1 The first line of defence

Probably the most important factor under this heading is the anatomical barrier to invading microorganisms provided by the epithelium. The purely morphological aspects of this barrier have already been discussed (see section 2.3) together with the defects which may predispose to candidal infections (see section 2.5).

The environment of the oral cavity contains saliva which constantly bathes the oral mucosa, and a bacterial flora present on the surface of the epithelium both of which aid in prevention of fungal overgrowth. The mechanical flushing effect of saliva on the tissues is supplemented by the action of constituents such as lysozyme, thiocyanate-dependent antibacterial systems and lactoferrin (MacFarlane & Mason, 1972) which have been shown to retard growth of both bacteria and fungi *in vitro*. Also present are specific anticandidal antibodies (Lehner, 1965) mainly of the IgA class and raised titres of these have been reported in patients with oral candidosis (Lehner *et al.*, 1972).
Microbial interference probably contributes significantly in reducing numbers of *Candida* present in the oral cavity, the suppressive mechanisms involving competition for nutrients (Knight & Fletcher, 1971) and the production of antifungal substances (Young et al., 1956). A further factor which may operate at the mucosal surface is the phagocytosis and extracellular killing of *C. albicans* by migrating polymorphs; the ability of leucocytes to kill *Candida* has been often demonstrated in vitro (Louria & Brayton, 1964; Lehrer & Cline, 1969).

The role of saliva and associated factors in the defence against *Candida* would seem a significant one. MacFarlane & Mason (1973) found 70% patients with Sjögren's syndrome to have history of oral candidosis although no increased incidence of infection of rat mucosa was obtained when xerostomia was induced by hyoscine treatment (Jones & Adams, 1970).

### 2.7.2 The second line of defence

This consists of nonspecific factors and the specific humoral and cellular immune responses to *Candida* antigens penetrating the mucosa. While the nonspecific immunity associated with phagocytic cells and inhibitory factors present in serum may contribute significantly to natural resistance to fungal infection, the high incidence of immunological skin reactions to *Candida* components (Shannon et al., 1966; Holti, 1966) suggests a specific acquired immunity may also play a role in keeping the fungus at a commensal level.

In the normal healthy human the presence of a weak pathogen in the tissues stimulates phagocytosis by polymorphonuclear leucocytes and monocytes (Salvin & Neta, 1972) and phagocytosis of *Candida* would seem to be enhanced by complement and specific anticandidal antibodies (Davies & Denning, 1972; Ishikawa et al., 1972). Once phagocytosed the fungal organisms are then killed by lysosomal enzymes (Lehrer & Cline, 1969). However Davies and Denning (1972) suggested mechanisms involving extracellular killing of fungi by polymorphs may be effective in host defence against candidal hyphae. The role of the macrophage in the phagocytic
defence against Candida is more difficult to define. While phagocytosis of C. albicans by macrophages has been demonstrated in vitro (Stanley & Hurley, 1969) only an initial suppression of fungal growth was achieved, the fungus surviving and continuing to grow; it even seems possible that macrophages act as vectors, aiding dissemination of the fungi. However Ozato and Uesake (1974) suggested that macrophages may co-operate with other systemic factors in the trapping and killing of yeast in vivo, and Winner (1972) found macrophages from infected mice to have a greater anticandidal activity than those from normal mice while Mackaness (1967) demonstrated such enhanced phagocytosis to be associated with the development of delayed hypersensitivity.

Of the various components present in normal serum, unsaturated transferrin has been shown to inhibit growth of C. albicans and other fungi by competing with the organisms for unbound iron present in the serum (Caroline et al., 1964; King et al., 1975). Iron is an essential nutrient for the fungi and King et al. suggested that the transferrin may diffuse through damaged epithelium into the superficial cells and bind the iron present thus making it unavailable to the fungi and preventing further growth. Other factors are present in serum which limit the growth of C. albicans. Louria and Brayton (1964) demonstrated a factor which inhibited growth of the fungus although Chilgren et al. (1967) showed that this factor clumped the organisms together rather than killing them, and that anticandidal antibodies were capable of inhibiting this clumping factor.

Resistance to opportunistic fungi involves more than phagocytosis or innate immunity. The development of enhanced resistance through such cellular expressions as delayed hypersensitivity would seem to be most influential in limiting infection by C. albicans and some 50-85% of the population show a delayed skin reaction to C. albicans (Shannon et al., 1966; Holti, 1966). While candidal infection is a frequent complication of defective cellular immunity associated with severe abnormalities, such as absence of thymus gland, (August et al., 1968; DiGeorge, 1968) it is also associated with less severe but more specific defects in expression
of delayed hypersensitivity (Chilgren et al., 1969; Kirkpatrick et al., 1971; Valdemarsson et al., 1973; Wells et al., 1972). The latter situation is exemplified by chronic muco-cutaneous candidosis which may be associated with one or more defects in the expression of delayed hypersensitivity. Lehner et al. (1972) arranged the immune defects observed in patients with chronic muco-cutaneous candidosis in six groups of progressive severity and showed that chronic oral candidosis for example, was accompanied by a more limited cell mediated defect than chronic candidosis involving both skin and mucosa. Finally it should be mentioned that a number of workers have cited evidence for the lack of response to Candida antigen being a secondarily acquired defect in long standing candidal infections in some patients (Imperato et al., 1968; Paterson et al., 1971; Kirkpatrick & Smith, 1974).

Humoral immunity does not seem to play a major role in host resistance to fungal infections. Subjects lacking antibodies have not been found to be over susceptible to candidosis (Rosen et al., 1966) and patients with systemic or muco-cutaneous candidosis normally have an intact humoral response (Kirkpatrick et al., 1971; Lehner et al., 1972). However circulating antibodies may interact with the mediators of the cellular immune response, and antigen-antibody complexes may enhance the activity of lymphocytes (Salvin and Neta, 1972) and of phagocytic leukocytes (see above). It has also been suggested that the antibodies may block immune receptors and so retard the development of hypersensitivity (Oppenheim, 1969).
Rationale

It must be clear to the reader that there are many aspects of the behaviour of *C. albicans* that are poorly understood, among these being the mechanism of invasion and the effects of *Candida* on the epithelial cells which it parasitizes. The work to be described in this thesis set out to examine at the cellular level both the invasion of oral epithelium by the fungus and the relationships established between the host epithelial cells and the parasite in oral candidosis.

The use of human or animal biopsy tissue for such investigations has a number of disadvantages. The use of human tissues raises the ethical consideration of unnecessary biopsy when smears will often adequately confirm diagnosis, while the recognition of *Candida* within the tissues that permits sure clinical diagnosis, makes sequential studies of the early stages of invasion rather belated. A review of the literature revealed that there are few animal models of oral candidal infection, the most defined model being in the rat (*Jones & Adams, 1970; Jones & Russell, 1973(a)*). However it appears that this is not an optimum model for ultrastructural studies since clinical diagnosis of infection is difficult (Russell - personal communication) and an investigation of the entire mucosa would prove tedious for electron microscopic studies from the point of view of sampling.

The use of a simple *in vitro* model system comprised of oral mucosa maintained in organ culture and infected with *Candida* overcomes many of these difficulties and controlled infection can provide abundant material for a study of invasion and the early phases of the establishment of oral candidal infection.
CHAPTER 3

THE ESTABLISHMENT OF THE MODEL SYSTEM

Part I

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- 3.1.1 Choice of suitable oral mucosal tissue
- 3.1.2 The culture and maintenance of oral mucosa

3.2 EXPERIMENT I: TO EXAMINE IN VITRO NEONATAL RAT MUCOSA INFECTED WITH C. ALBICANS
- 3.2.1 Materials and Methods
  - 3.2.1.1 The culture laboratory
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  - 3.2.1.3 Preparation of the culture units
  - 3.2.1.4 Animals
  - 3.2.1.5 Preparation and maintenance of the explants
  - 3.2.1.6 The fungus and preparation of inoculum
  - 3.2.1.7 Infection and harvesting of explants
  - 3.2.1.8 Fixation and preparation for light microscopy
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  - 3.2.2.1 Assessment of the control explants
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Part II

3.4 INTRODUCTION
3.5 EXPERIMENT II: TO STUDY THE EFFECTS OF ANTIBIOTICS AND CORTICOSTEROIDS ON THE MODEL SYSTEM
- 3.5.1 Materials and Methods
  - 3.5.1.1 Nutrient media
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3.6 DISCUSSION

3.7 CONCLUSIONS
3.1 INTRODUCTION

In order to make any valid comparisons between the host-parasite relationship established between C. albicans and oral epithelium in vitro and in vivo, the cultured host tissue must be maintained as close to the normal state as possible. This requires precise culture methods known to provide optimal maintenance of the explanted tissues. Infection of the culture system with C. albicans might be expected to have deleterious effects on the explanted tissue especially if the fungus is allowed to proliferate excessively; therefore the amount of Candida introduced initially into the system should be minimal so as to reduce the effects of its metabolic by-products on the cultured tissues. There are thus three prerequisites for the establishment of the model system:

a) The choice of suitable oral mucosal tissues
b) The successful culture and maintenance of oral mucosa
c) The infection of the selected tissues with C. albicans

3.1.1 Choice of suitable oral mucosal tissue

Neonatal animal tissues have been successfully maintained in vitro and provide the most suitable choice of tissue for a pilot study (see next section). At the same time it would seem reasonable to choose a tissue which has been shown to be susceptible to candidal infection. The dorsal mucosa of rat tongue, which is covered by an orthokeratinized epithelium, has been successfully infected with C. albicans in vivo in both the adult rat (Jones & Adams, 1970) and the neonate (Jones & Russell, 1973b).

3.1.2 The culture and maintenance of oral mucosa

Although a number of investigations into the optimal conditions for culturing skin have been described including those by Iverson (1968);
Siddiqui et al. (1974); Gaylarde and Sarkany (1975); Flaxman and Harper, (1975) definitive studies of in vitro maintenance of oral mucosa have only recently been made (Chang & Maibach, 1967; Bergenholtz, 1969; Miles, 1974; Hill & Miles, 1976). The techniques which have been used for in vitro maintenance of skin and mucosa vary widely, particularly with regard to the nature of the media, the composition of the gas phase and the incubation temperature. No one method seems entirely successful although Hill (1976) has recently maintained rat palatal mucosa successfully in vitro for up to 28 days and mentions several conditions which do seem to be important for maintenance. These are:

a) Positioning of the tissue explant at a gas-liquid interphase
b) The use of a chemically defined nutrient medium - in the past the majority of workers have used natural media, or have incorporated 10-20% serum in synthetic media. This does not now seem to be obligatory for the survival of skin and mucosa
c) Selection of an appropriate gas phase - the optimal concentration of oxygen for oral mucosa was shown to be approximately 50% (Melcher & Hodges, 1968; Bergenholtz, 1969; Miles, 1974; Gerson, 1975)

An evaluation of the maintenance of mucosa and skin in vitro has been, and remains, somewhat subjective and histological assessment of the tissue morphology and its preservation with time reveals little information about the functional properties of the tissue for it is well known that degenerative changes occur at a molecular level long before morphological manifestations become apparent. Ultrastructural studies have been able to demonstrate changes taking place at a subcellular level (Sarkany & Gaylarde, 1970; Bergenholtz, 1969; Hill, 1978).

Major factors which limit the survival of explants would seem to be:

a) Pre-existing disease states in the tissues (Sarkany et al., 1965)
b) The degree of insult imparted during tissue removal and the condition of the tissues at the start of maintenance in culture (Hill, 1976)
c) The time delay in explantation (Sarkany et al., 1965)
The use of experimental animals minimises the problem of diseased tissue, and the most favorable results have been obtained with such animal tissues (Bergenholtz, 1969; Bergenholtz & Theilander, 1970; Miles, 1974; Hill & Miles, 1976). Hill has shown that neonatal tissues are maintained more successfully than adult tissues, possibly as a consequence of their greater potential for growth and differentiation.

The techniques of organ culture and methods of in vitro maintenance used in the studies to be described are based upon those of Miles and Hill who maintained rat mucosa in vitro for 10 days and for 28 days respectively. Details of the methods are given later in the chapter.

3.2 EXPERIMENT 1: TO EXAMINE IN VITRO NEONATAL RAT MUCOSA INFECTED WITH C. ALBICANS

3.2.1 Materials and Methods

3.2.1.1 The Culture Laboratory

A special room was utilised for the setting up and maintenance of the explants. The design was such as to minimise falling infection and the stirring up of dust or micro-organisms by draughts. This culture laboratory was used solely for organ culture and thus chemical contamination was reduced. All personnel using this area wore caps, masks and gowns to prevent contamination of the working area. Glassware, and all instruments used were sterilised either in hot air for 2 hours at 160°C. or autoclaved at 15 lbs/sq. inch for 15 minutes and stored separately in capped containers, the culture units were all maintained packaged in the laboratory. All manipulation of tissues was carried out within a laminar flow unit (Microflow Ltd. Hants), previously swabbed with 70% alcohol.

3.2.1.2 Nutrient Medium

The nutrient media was prepared from Waymouth's MB 752/1 dried medium (Wellcome Reagents Ltd. Kent.) supplemented with 0.825 μg/ml ferrous sulphate (FeSO₄. 7H₂O); 300 μg/ml ascorbic acid; 100 μg/ml streptomycin sulphate (Dista Products Ltd.); 120 μg/ml benzyl penicillin
(Dista Products Ltd.) and 1 μg/ml hydrocortisone sodium succinate. It was titrated to pH 7.35 with 1M sodium hydroxide and buffered with sodium bicarbonate (2.2 mg/ml). The medium was sterilised by passing through a Millipore filter (pore size 0.22 μm), bottled in 25 ml aliquots and stored at -20°C. until ready for use.

3.2.1.3 Preparation of the culture units

The sterile culture units consisted of a large outer plastic petri dish (Nundon Δ) containing a lining of filter paper moistened with normal saline to maintain a humid environment for the explants. Inside the dish were placed 30 mm diameter tissue culture quality plastic petri dishes (Nunclon Δ) which contained the culture platforms, approximately 15 mm by 15 mm and 3 mm high, made from stainless steel "Minimesh" (The Expanded Metal Co. West Hartlepool) after Trowell (1954).

About 1.5 ml of nutrient medium was placed in the plastic petri dishes, the amount of medium being just sufficient to wet the platform of the grid - minimal wetting of the undersurface of the explants is reported to be an important factor for their successful culture (Miles, 1974). If the explants were to be maintained for longer than 48 hours, the nutrient medium was changed at this time.

3.2.1.4 Animals

The animals used in these experiments, together with all the neonates used in the following studies were bred and maintained in the animal house of the London Hospital Dental Institute. In the present experiments the tissues were taken from Sprague-Dawley rats 0-4 days old.

3.2.1.5 Preparation and maintenance of the explants

The rats were decapitated and the heads were placed in Tyrode solution (Difco Labs) to which 120 μg/ml of benzyl penicillin and 100 μg/ml streptomycin sulphate had been added. The lower jaw and tongue were dissected from the upper jaw and stored in fresh Tyrode solution. Tongue explants were prepared by pinning the mandible on to silicone rubber under a dissecting microscope the tongue was incised horizontally anterior to the circumvallate papillae and foramen caecum just deep to the epithelium.
so that forward movement of an iridectomy knife "shaved" off a thin explant of the dorsal epithelium. This was divided to produce a number of explants 1-2 mm square and 1-1.5 mm thick which were trimmed and washed in a cavity slide containing Tyrode solution, before being re-washed in culture medium and then mounted, epithelium uppermost, on small squares of Millipore filter type HA (pore size 0.45 μm).

The culture units were placed in a McIntosh and Fildes jar and gassed with 55% O₂, 40% N₂, 5% CO₂ (British Oxygen Corp.) for at least ten minutes at a flow rate of 2 litres per minute, sealed and incubated at 37° C.

3.2.1.6 The fungus and preparation of inoculum

Details of the source, identification and maintenance of the strains and species of Candida used in all the following studies are given in Appendix I.

A 24-hour colony of C. albicans (Strain 3091A) grown on Sabouraud's dextrose agar (Oxoid Ltd., London) at 37° C. was suspended in 1% peptone water (Oxoid Ltd.). The optical density of the suspension was read in a spectrophotometer (S.P. 500; Pye Unicam Ltd., Cambridge) at a 400 nm wavelength, and the number of cells per microlitre of fluid was calculated from a standard reference curve, prepared by plotting the optical densities of different dilutions of a yeast suspension against viable counts of each dilution by the method of Miles, Misra and Irwin (1938). The yeast suspensions could thus be diluted to give the required number of viable cells per microlitre of fluid.

3.2.1.7 Infection and harvesting of explants

Twenty-one explants of tongue were prepared and maintained in vitro for approximately 4 hours. Fourteen explants were then inoculated with 10-20 viable yeast cells of C. albicans in 1 μl of fluid by means of sterile 1 μl Microcap pipettes (Drummond Scientific App. U.S.A.), the remaining seven explants acting as uninfected controls. After inoculation the original maintenance conditions were resumed and the tissues were
<table>
<thead>
<tr>
<th>Dorsal mucosa of rat tongue</th>
<th>Medium</th>
<th>Nos. of Explants</th>
<th>Incubation Period in Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T.C.M.</td>
<td>Controls</td>
<td>Infected</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
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</table>

Fig. 3.1 Dorsal mucosa of neonatal rat tongue maintained in vitro for 39 hrs. The organization of the tissue shows few changes from in vivo, a thickened keratin layer is present due to accumulation of squames on the tissue surface. x 360 (p.a.S., haematoxylin).
harvested in groups of three explants (two infected and one control) at intervals between hours 18 and 39 hr. (see Table 3.1).

3.2.1.8 Fixation and preparation for light microscopy

Tissues were fixed in Zenker acetic for 45 mins., washed and post fixed in Zenker stock for 1 hour (Wallington, 1955), dehydrated and embedded in paraffin wax. Serial sections (5 µm thick) were stained by periodic-acid-Schiff (p.a.S.) after McManus (1946) to demonstrate the presence of Candida and counterstained with Erhlich's haematoxylin or tartrazine.

3.2.2 Results

3.2.2.1 Assessment of the control explants

The dorsal mucosa of neonatal rat tongue is covered by a stratified squamous orthokeratinizing epithelium that is organised into discrete filiform papillae showing the so-called dual pattern of keratinization (Farbman, 1970). These papillae are supported by a loose connective tissue, beneath which are the muscle fibres of the tongue. Generally this structure was well maintained in vitro (fig. 3.1) although, as would be expected, there were variations in tissue maintenance between different regions of the same explant and also between different explants. The central region of the explant maintained its overall structure well, and mitotic figures were seen in both basal and suprabasal epithelial cells. The margins of the explants showed some disorganization, the epithelium being reduced to a single flattened nucleated layer covered by a thin layer of squames, and migration of the epithelium over the surface of the connective tissue (epiboly) was beginning.

An accumulation of keratin squames on the surface of the explants was noticeable even after only 24 hours of maintenance (fig. 3.1), and was probably due to the lack of abrasive removal that normally takes place in vivo. The maintenance of the connective tissue and muscle was more difficult to assess by light microscopy but variations in the appearance of the tissues could be seen including areas of patchey
Fig. 3.2  Rat tongue mucosa - 26 hours after inoculation with *C. albicans*, showing a colony of fungus growing on top of the epithelium, and possible hyphal penetration of the superficial cells. x 140.

Fig. 3.3  Penetration of the cells of the granular layer by hyphae of *C. albicans* after 24 hours *in vitro*. x 200.
acellularity and degeneration of muscle fibres. In some explants large vacuoles were present in the deeper regions of the connective tissues; it has been suggested that this reflects the level reached by the nutrient medium around the explant and represents a zone of water logging (Hill-personal communication). Nevertheless such changes in the deep tissues did not seem to have deleterious effects on the overlying epithelium.

3.2.2.2 Assessment of infection with \textit{C. albicans}

In the experiments in which inocula containing about 10 yeast cells were employed, all of the inoculated explants showed evidence of infection and colonies of \textit{C. albicans} were seen to grow on top of the tissues (fig. 3.2). Blastospores and mycelial forms of the fungus were present and by 18 hours the M-phase could be seen penetrating the superficial keratin layer. As the incubation time increased, the penetration of the tissues progressed, so that by 24 hours fungi could be seen in the granular layer of the epithelium (fig. 3.3), by 26 hours in the prickle cell layer and by 28 hours in the basal cell layer (fig. 3.4). After 30 hours of incubation, infection of the explants had become extensive, the hyphae having passed completely through the epithelium so as to deeply penetrate the connective tissues (fig. 3.5). The predominant fungal form present within the tissues was the M-phase, although the Y-phase was also seen where tissue invasion was heavy. However, the Y-phase was never present in isolation in any of the cell layers.

Examination of the tissue structures showed that superficial invasion of the epithelium had no deleterious histological effects on the deeper tissues. Where invasion by hyphae was more extensive, some disruption of the organization of the epithelium was seen, as will be discussed more fully in Chapter 5.
Fig. 3.4  Penetration of the basal cell layer of tongue epithelium by the *C. albicans* after 28 hours *in vitro*.  x 875.

Fig. 3.5  Fungal invasion through the basement membrane into the connective tissues after 39 hours *in culture*.  x 350.
THE ESTABLISHMENT OF THE MODEL SYSTEM: PART (II)

3.4 INTRODUCTION

In developing the model system, one further factor needs to be considered and that is the effect of the culture system on the proliferation of the fungus. Previous studies have demonstrated that *C. albicans* will readily proliferate in both the Y-phase and M-phase in tissue culture media incubated at 37°C and that the presence of penicillin, streptomycin and cortico-steroids in the media did not appear to effect the proliferation rate or fungal form (Howlett, 1973). Nevertheless it was felt that the best way to assess the effect of these drugs on the growth and pathogenicity of *C. albicans* was in the culture system itself. While antibiotics are necessary to prevent overriding infection by the contaminating oral flora, it should be possible to maintain embryonic tissue, removed and cultured under sterile conditions without the use of antibiotics. Therefore for this particular experiment embryonic animals were used although their tongue mucosa is not as fully developed (Miles, 1974) and it is not so convenient to use nor as dependable in a culture system as those of neonatal tissues.

3.5 EXPERIMENT II: TO STUDY THE EFFECTS OF ANTIBIOTICS AND CORTICO-STEROIDS ON THE MODEL SYSTEM

3.5.1 Materials and Methods

3.5.1.1 Nutrient media

Four different nutrient media were prepared:

i) Tissue culture medium as used in experiment (1).

ii) Tissue culture medium without penicillin and streptomycin.

iii) Tissue culture medium without hydrocortisone.

iv) Tissue culture medium without penicillin, streptomycin and hydrocortisone.

Sterilisation and storage of the media were carried out as in experiment (1).
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Media</th>
<th>Numbers of Explants</th>
<th>Incubation Period</th>
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<tr>
<td></td>
<td></td>
<td>Infected</td>
<td>Controls</td>
</tr>
<tr>
<td>Neonatal rat tongue</td>
<td>a) T.C.M.</td>
<td>2</td>
<td>1</td>
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<td></td>
<td>2</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td>3*</td>
<td>1*</td>
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<tr>
<td></td>
<td>b) T.C.M. without hydrocortisone</td>
<td>2</td>
<td>1</td>
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<td></td>
<td></td>
<td>2</td>
<td>1</td>
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<td>3*</td>
<td>1*</td>
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<tr>
<td>Embryonic rat tongue</td>
<td>a) T.C.M.</td>
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<td></td>
<td></td>
<td>3*</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>b) T.C.M. without antibiotics</td>
<td>2</td>
<td>1*</td>
</tr>
<tr>
<td></td>
<td>c) T.C.M. without hydrocortisone</td>
<td>2</td>
<td>1*</td>
</tr>
<tr>
<td></td>
<td>d) T.C.M. without antibiotics or hydrocortisone</td>
<td>4</td>
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<td>4*</td>
<td>2*</td>
</tr>
</tbody>
</table>

*Denotes each explant bisected and half fixed and processed for electron microscopy.
3.5.1.2 Source and infection of tissues

A Sprague-Dawley rat in the 20th day of pregnancy was killed by ether inhalation and the embryos were removed aseptically. Explants from the dorsal mucosa of the embryos' tongues together with those from the neonatal rats' tongues were prepared, maintained, and infected with approximately 30 yeast cells of *C. albicans* as in experiment (1), according to the regime set out in Table 3.2.

3.5.1.3 Fixation and preparation of tissues

Specimens for light microscopy were fixed and processed as in experiment (1).

Specimens for electron microscopy were fixed with buffered gluteraldehyde and post-fixed with osmium tetroxide. Full details of the methods used for electron microscopy are described and discussed in Chapter 6.

3.5.2 Results

On histological examination of the neonatal tongue explants, no difference could be seen between those explants maintained in media with or without hydrocortisone. The maintenance of the epithelium was good in all explants and organization of the tissues was similar to that described in experiment (1). Explants from embryonic rat tongue showed some variation in the degree of development of the filiform papillae; this is in accord with Miles (1974) who found full development of the filiform papillae present only on the anterior half of rat embryo tongue. Maintenance of explants cultured for a maximum of 48 hours was generally satisfactory, the only signs of tissue disturbance being in the connective tissue at the edges of the explants as in the previous experiment. No obvious differences could be seen between tissues maintained by the different media and no bacterial infection occurred in the explants cultured without antibiotics. Ultrastructural examination of both neonatal and embryonic tissues confirmed that the subcellular organization of tissues was not effected by the presence of antibiotics or hydrocortisone (fig. 3.6, 3.7).
Fig. 3.6(a) An electron micrograph showing the epithelium from the dorsal mucosa of embryonic rat tongue, from an explant maintained in vitro for 30 hours in tissue culture media with antibiotics and cortisone. x 4,000.

(b) Epithelium from an embryonic tongue explant maintained in media without antibiotics and cortisone. x 4,000.

(Tissue sections stained with uranyl acetate and lead citrate).
Fig. 3.7(a) Epithelial cells of the basal layers of neonatal rat tongue mucosa maintained in vitro for 30 hours with tissue culture media containing antibiotics and cortisone. x 5,000.

(Tissue sections stained with uranyl acetate and lead nitrate).

(b) The basal cells of neonatal mucosa maintained for 30 hours with media containing no cortisone. x 5,000.
The explants infected with *C. albicans* showed proliferation of the fungus in both the Y-phase and M-phase. The fungi penetrated the epithelium and often, in both neonatal and embryonic explants, reached the connective tissues; however penetration of embryonic tissues did appear to take place at a faster rate than that of neonatal explants. Again, no obvious differences could be seen in either the activity of *C. albicans* or of the tissue response in the explants maintained in the different media, and this uniform response was confirmed by ultrastructural examination of the specimens. (The ultrastructure of the relationship between *C. albicans* and the oral mucosa will be described in Chapter 6).

### 3.6 Discussion

The two pilot experiments described in this chapter set out to establish conditions for an *in vitro* model of oral candidosis. The results indicate that conditions which allow for the invasion of oral epithelium maintained *in vitro* by *C. albicans* were achieved. The events taking place in the *in vitro* system are sufficiently similar to those occurring *in vivo* (Jones & Adams, 1970) to encourage further evaluation of the model. However candidal infection of the oral mucosa *in vivo* is generally limited to the superficial epithelial layers but in the present model as in previous *in vitro* systems (Blank et al., 1959; Pemberton & Turner, 1973) deep invasion of the epithelium and connective tissues does take place. This raises significant questions about the model system. For example are there factors present only *in vivo* which restrict fungal penetration of the tissues or does the deep invasion *in vitro* reflect a lack of structural integrity in the cultured tissues, so that they are merely serving as growth media for the fungus?

The methods of organ culture used here to prepare and maintain the explanted mucosa are a reliable means of providing tissue to act as host to fungal invasion. A number of histological criteria have been proposed for evaluation of epithelial explants (Sarkany & Gaylarde, 1970) and these include:
i) preservation of the epithelial architecture
ii) presence of mitotic figures in the basal layers
iii) continued formation of the keratin layer
iv) proliferation of the epithelium from the free edge of the explant.

Hill (1976) has also emphasised the role of connective tissue maintenance in the survival of the tissues.

The explanted tissues examined in the present experiments generally fulfilled all these criteria. Although some disorganization was apparent in the connective tissues and at the explant edges, the epithelial structure as a whole was well preserved; this is important for it is the epithelium which is invariably the host to fungal infection in oral candidosis.

The method used to infect the explants with \textit{C. albicans} in the model system was very satisfactory. The micro-inoculum used in experiment (II) contained approximately 30 viable yeast cells which is considerably less than that used either by Blank et al. (1959) in dermatophyte infection of human skin \textit{in vitro} or by Pemberton and Turner (1973) in candidal infection of human gingiva. Nevertheless all inoculated explants showed evidence of infection and invasion by \textit{C. albicans}, and propagation of the fungi under the \textit{in vitro} conditions was rapid. However the rate of fungal penetration of the tissues was less than that reported by Pemberton and Turner (1973) who stated that initial penetration of the epithelium took place after 6 hours, and deep penetration to the spinous layers occurred by 18 hours. In the first pilot experiment, described above, fungal invasion was limited to the keratin layer for a relatively long period of time (approximately 24 hours), but once penetration of this layer was achieved the movement of the fungal hyphae through the remaining epithelial layers and into the subepithelial tissue was rapid. These differences in the speed of invasion may reflect the difference in inoculum size or alternatively the condition of the host tissues. Pemberton and Turner were using adult human gingiva which cannot usually be maintained as satisfactorily \textit{in vitro} as neonatal animal mucosa (Hill, personal communication). Penetration of the host tissues by the fungi in experiment (II)
was more rapid and may well reflect the slightly larger inoculum dose or the use of embryonic tissue. Miles (1974) has pointed out that structural organization of the epithelium is not complete in embryonic rat tongue so that the resistance of the epithelium to infection may be lower than in the neonate.

The concentrations of antibiotics present in nutrient media have been shown to be without deleterious effects on human skin in vitro (Cruikshank & Lowbury, 1952) and are in general use in culture systems (Paul, 1975). There has always been concern over the influence of antibiotics on fungal behavior in vivo, for penicillin and streptomycin are thought to encourage candidal infection, but by the inhibition of competitive bacterial flora, rather than by any direct effect on the fungi themselves (see section 2.5). In the present experiments no difference could be seen in the maintenance or extent of infection of the explants cultured with or without these antibiotics. As neonatal tissues would be likely to succumb to bacterial infection due to contamination from their environment, this finding allows for the use of antibiotics in the culture system.

Hydrocortisone is a frequent constituent of tissue culture media and is incorporated in the present system according to Miles (1974) and Hill (1976), although neither of these workers have made definitive studies of its effect on the system. Hydrocortisone has been shown to stabilise lysosomal enzymes (Weissman, 1969) and has been claimed by Sarkany and Gaylarde (1970) to extend the viable culture period. These latter workers also reported that the steroid was able to promote keratinization of epidermis in vitro, suppress mitotic activity and diminish migration of epithelial cells. The influence of corticosteroids on infection by C. albicans has been mentioned in Chapter 2 (section 2.5) and generally these agents facilitate fungal invasion by a suppression of the immune response rather than by directly affecting the growth of the organism itself. Thus hydrocortisone might be expected not to influence the activity of C. albicans and in the pilot experiments no differences could be seen in the infection of tissues with or without hydrocortisone. On
the other hand steriods might influence the maintenance of the tissues in vitro or affect their response to fungal invasion, however no effects were observed on the tissues in either the histological or electron micro-scopical studies although the ultrastructural studies were not extensive enough, nor was the duration of the in vitro maintenance sufficiently long enough to reveal minute or latent changes.

3.7 CONCLUSIONS

There was thus little evidence from the pilot studies to suggest that either antibiotics or hydrocortisone adversely affected the model system of oral candidosis and it was decided to continue using these constituents relying on the unproven assumptions of other workers in the field that the agents may be doing undiscerned good! However Raisz (personal communication) pointed out that the level of steroid employed in the system may be higher than that normally present systemically so that in all subsequent experiments the level of cortisone was cut to half that used in these pilot studies.
CHAPTER 4
THE EVALUATION OF THE MODEL SYSTEM

4.1 INTRODUCTION

4.2 EXPERIMENT III: THE INFECTION OF NON-KERATINIZED AND PARA-KERATINIZED MUCOSA FROM NEONATAL RABBIT WITH C. ALBICANS

4.2.1 Introduction
4.2.2 Materials and Methods
4.2.2.1 The inoculation techniques
4.2.2.2 Preparation and infection of the explants
4.2.2.3 Fixation and processing
4.2.3 Results
4.2.3.1 Assessment of the control explants
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4.3.1.1 Fixation and processing
4.3.2 Results
4.3.2.1 Histological assessment of keratinized explants
4.3.2.2 Histological assessment of non-keratinized explants
4.3.2.3 Assessment of infected tissues by scanning electron microscopy
4.3.3 Discussion

4.4 CONCLUSIONS
4.1 INTRODUCTION

The pilot studies described in Chapter 3 demonstrate that similarities exist between the invasion of keratinized mucosa in vitro and in vivo, but they also raise a number of questions about the behavior of both the fungi and the explanted tissues. In addition, the model only reveals the activity of one strain of C. albicans even though the virulence of strains of C. albicans has been shown to differ somewhat (see section 2.6.3). Thus although no great differences in the pathogenicity of non-variant strains exist it does seem necessary to compare a number of different strains to verify the behavior of the specimen in the model system.

It seems unlikely that keratinized rat tongue mucosa should be more susceptible to infection by Candida than other types of mucosa. While the use in the in vitro system of epithelia showing other degrees of keratinization would increase the value of the model, for many superficial infections are established in epithelia which are initially non-keratinized or para-keratinized. Therefore as the oral mucosa of rat is orthokeratinized another species is necessary to provide non-keratinized and para-keratinized tissues. Of the non-primates the rabbit has non-keratinized buccal mucosa which is closest in structure to the non-keratinized human mucosa (Chen, 1969). Neonatal rabbit cheek mucosa has been maintained in vitro by Gerson (1974) using methods similar to Miles (1974). Mucosa from the dorsum of the neonatal rabbit tongue is para-keratinized, the nuclear remnants being visible histologically in the most superficial epithelial cells. There is also evidence in the literature of an experimental oral candidosis in rabbit (Mackinnon, 1936, cited in Winner & Hurley, 1964). Thus neonatal rabbit cheek and tongue mucosa appear to be suitable host tissues for fungal infection in vitro, and the first experiments described in this chapter examine the behavior of these tissues in the model system.

The second phase of the evaluation of the model system will consider the integrity of the host tissue during fungal infection. One of the most
striking features described in the previous chapter is the rapid and extensive growth of the fungus in the cultured tissue. This was such as to suggest that the tissue might be acting merely as a convenient growth medium in which the fungus can proliferate. To test this possibility experiments will be described which compare the invasiveness of different species of Candida which show a range of pathogenicity, as determined by their cytopathic effects on epithelial cells both in culture (Stanley & Hurley, 1969) and in vivo (Hurley, 1966). If the cultured tissues are merely acting as a passive growth medium then it might be expected that all species would show a similar invasiveness.

4.2 EXPERIMENT III: THE INFECTION OF NON-KERATINIZED AND PARA-KERATINIZED MUCOSA FROM NEONATAL RABBIT WITH C. ALBICANS

4.2.1 Introduction

The initial investigations in this series employed a similar inoculation system to that used in the previous experiments (Chapter 3) and while producing an acceptable infection of the para-keratinized tongue explants it provided little infection or fungal proliferation on the surface of non-keratinized explants. Consequently two further methods of inoculation were developed in order to produce satisfactory infection of the non-keratinized explants.

4.2.2 Materials and Methods

4.2.2.1 The inoculation techniques

The strain of C. albicans used in these experiments was strain 'T' (see Appendix 1).

Two methods of inoculation of explants were used:

a) a fluid micro-inoculation as in experiment (1) containing approximately 25 or 50 viable yeast cells in 1 µl of fluid. In some cases a sterile gas chromatogram syringe was used to distribute 30 yeast cells in 0.3 µl of fluid.

b) inoculation with a micro-colony; pieces of very fine chrome/nickel wire were sterilised and used to inoculate the explants.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Inoculation dose</th>
<th>Nos. of Explants</th>
<th>Incubation period in hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cheek</td>
<td>1 µl containing approx. 30 cells</td>
<td>1 infected explant 1 control for each time period</td>
<td>16, 18, 22, 28</td>
</tr>
<tr>
<td>Cheek</td>
<td>1 µl containing approx. 50 cells</td>
<td>2 infected explants and 1 control for each time period</td>
<td>20, 30, &amp; 40</td>
</tr>
<tr>
<td>Tongue</td>
<td>1 µl containing approx. 50 cells</td>
<td>2 infected explants and 1 control for each time period</td>
<td>20, 30, &amp; 40</td>
</tr>
<tr>
<td>Cheek</td>
<td>0.3 µl containing approx. 30 cells</td>
<td>4 infected explants and 1 control</td>
<td>30</td>
</tr>
<tr>
<td>Tongue</td>
<td>0.3 µl containing approx. 30 cells</td>
<td>4 infected explants and 1 control</td>
<td>30</td>
</tr>
<tr>
<td>Cheek</td>
<td>micro-colony applied by wire inoculation</td>
<td>4 infected controls and 1 control</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 infected explants and 1 control</td>
<td>15</td>
</tr>
</tbody>
</table>
Under a dissecting microscope one end of the wire was touched against a colony from a fresh 24 hour growth of *C. albicans* and then touched lightly against the surface of the explant.

**4.2.2.2 Preparation and infection of the explants**

The tissues were obtained from 0-2 day old New Zealand white rabbits. The animals were asphyxiated with carbon dioxide, decapitated and the tongue and anterior portion of the lower jaw were dissected from the head, care being taken to preserve the cheek mucosa adjacent to the molar teeth.

The cheeks, together with the underlying skin and supporting areas of the lower jaw and maxilla were dissected away from the rest of the head. These tissues were pinned out on silicone rubber to separate the molar teeth and reveal the non-keratinized buccal mucosa of the cheek. This was dissected off the underlying tissues under a microscope using iridectomy scissors, care being taken not to disturb the fragile surface of the non-keratinized epithelium. Excess supporting tissue, muscle and fat were trimmed away, and the tissue was then cut into 4-6 explants 1-2 mm square using an iridectomy knife. The explants were generally a little thicker than the tongue explants, being about 1.5-2 mm thick.

The tongue explants were prepared as described for rat tongue. All tissues were maintained with normal nutrient media and under conditions described in experiment (1). The explants were inoculated with *C. albicans* within 24 hours of explantation, as indicated in table 4.1.

**4.2.2.3 Fixation and processing**

Explants were harvested at times shown in table 4.1, fixed and processed for light microscopy as before.
Fig. 4.1 The dorsal mucosa of neonatal rabbit tongue maintained in vitro for (a) 30 hrs. (b) 40 hrs., showing the variation in the degree of keratinization seen in explants of this tissue. x 340 (p.a.S., haematoxylin).

Fig. 4.2 Neonatal rabbit cheek mucosa maintained in vitro for (a) 20 hrs. (b) 15 hrs., showing the variable thickness of the epithelium across the cheek mucosa. x 350.
4.2.3 Results

4.2.3.1 Assessment of the control explants

a) Tongue

The dorsal tongue explants from neonatal rabbit were successfully maintained in vitro, and their organization and preservation were generally similar to the explants of rat tongue. Some differences were noted however; rabbit epithelium was thicker and had a greater number of cell layers than that of rat tongue (fig. 4.1). In most cases the epithelium was para-keratinized, and conspicuous pyknotic nuclei were present in the surface squames. On rare occasions complete keratinization was seen (fig. 4.1).

b) Cheek

Successful maintenance of rabbit cheek mucosa in vitro proved more difficult than that of tongue. This may be the reason for the paucity of reports in the literature describing successful culture of non-keratinizing oral mucosa. Some of the explants in the first experiments did not maintain their structure well, a lack of cohesion between the cells of the outer layers of the epithelium being the most obvious disturbance. However with more care in the preparation and the handling of the explants the preservation of the tissues reached a satisfactory level.

On histological examination the stratification of the epithelium of non-keratinized mucosa did not appear as well defined as that of keratinized mucosa (fig. 4.2). Basal and para-basal cells, with large rounded nuclei were situated above a distinct basement membrane and cells undergoing mitosis were clearly visible in this region. A thick spinous cell layer was present, the cells of which gradually increased in size and decreased in staining intensity so that in the upper spinous layers they often had a somewhat vacuolated appearance. Glycogen, which in these layers stained intensely with the periodic acid Schiff reaction, was abundant in these layers and persisted in the superficial cells; no great change in the distribution or amount of glycogen was visible during
Fig. 4.3  Neonatal rabbit tongue mucosa infected with a small colony of *C. albicans* by means of wire, and maintained *in vitro* for 12 hrs. Candidal hyphae are seen deeply invading epithelium. x 250.

Fig. 4.4  Neonatal rabbit cheek mucosa infected with a small colony of *C. albicans* by means of a wire and maintained *in vitro* for 15 hrs. x 250.
the culture period. The prickle cells gave way to moderately flattened, basophilic cells with elongated nuclei containing condensed chromatin. In some biopsies some condensation of superficial cells was evident giving the appearance of para-keratinization, while in others the superficial cells layers seemed to lie unattached on the surface of the explants, perhaps representing cells which would have been desquamated in vivo.

While the above description applies to the non-keratinized mucosa in general a marked variation in both the thickness of the epithelium and in the number of cell layers constituting the various strata was seen from one part of the cheek mucosa to another (fig. 4.2).

The underlying connective tissue and muscular submucosa showed some signs of degeneration as did the explants of tongue mucosa. Glandular elements were often present in the cheek tissues, the epithelial elements of which retained their organization. Both epithelium and connective tissue showed some degeneration at the cut edges of the explant, many of the cells containing pyknotic nuclei and vacuolated cytoplasm.

4.2.3.2 Assessment of the infected explants

a) **Tongue**

All of the infected tongue explants were invaded by *C. albicans*. Both Y-phase and M-phase of the fungus were present, proliferating on the tissues and within them, although the M-phase predominated in the latter site (fig. 4.3). In those explants infected with a fluid inoculum, proliferation of *Candida* at the explant edges and subsequent invasion of the adjacent connective tissues was seen. Fungal penetration of the epithelium occurred more rapidly than in rat tongue mucosa, and in one of the explants fungal invasion of the entire epithelium occurred within 24 hours. Reduction of the amount of liquid used to distribute the yeasts to 0.3 μl in order to reduce the flow of inoculum over the edge of the explant did not increase the incidence of surface colonization by *C. albicans* nor affect the pattern of infection of the tissues.

Infection of the explants by means of fine gauge wire proved successful in all cases. No trauma to the host tissues from the wire was
evident histologically and large colonies of yeasts were present on the surface of the epithelium at the sites of inoculation. Proliferation of both Y-phase and M-phase of *C. albicans* from the edges of the colony was often heavy, especially in those explants incubated for 15 hours after inoculation. Penetration into epithelium as far as the deeper epithelial cell layers often occurred by 12 hours, and reached the connective tissue after 15 hours. Heavy proliferation of fungi at the explant edges did not occur in tissues infected by wire inoculation.

b) Cheek

The incidence of infection of cheek explants by the fluid inoculum, which had proved successful for tongue explants, was low. The 16 hour specimen showed little evidence of infection, and those of 18, 22 and 28 hours showed only invasion of tissue at the explant edges. Increased inoculation doses and longer periods of incubation still did not produce a high incidence of surface infection or invasion of epithelium, although proliferation of *Candida* at explant edges allowed the hyphae to invade the exposed subepithelial tissues and to spread through the basement membrane to the deeper epithelial cell layers.

When a smaller fluid inoculum was used two of the four explants showed a colony of *C. albicans* growing on the epithelial surface and invasion of the underlying tissues. However in every explant inoculated by means of wire surface colonization and invasion of the epithelial tissues by *C. albicans* was seen (fig. 4.4). Because of the difference in thickness of the epithelium in explants from different regions of the cheek mucosa it is difficult to compare rates of epithelial penetration. However in those explants maintained for 12 hours after infection, hyphae of *C. albicans* had invaded the epithelial tissues, penetrating through about half the epithelial layers in the explants with a thick epithelium, and through the whole of the epithelium in the explants with a thinner epithelium. A heavier invasion was seen in those explants maintained for 15 hours after inoculation.
4.2.4 Discussion

Successful maintenance of the non-keratinized tissues in vitro was achieved by increased care during the preparation of explants while successful inoculation of these tissues with *C. albicans* required the use of a procedure other than that used for tongue mucosa. Whether the absence of surface proliferation and invasion by *C. albicans* when the non-keratinized explants were infected with the liquid inocula implies some natural tissue resistance to fungal colonization on the part of this tissue or merely reflects the difficulty for the yeasts in gaining a purchase on the smoother convex surface of the non-keratinized explants as compared to the papillate surface of tongue explants, cannot be stated. However it seems that in future experiments the wire inoculation technique must be used if satisfactory infection of the non-keratinized explants is to be obtained with any regularity. Certain disadvantages may arise from this as a larger number of yeasts and their metabolic products will be present in the model system and may interfere with the maintenance of the explants. However the infection of the tissue proceeds more rapidly using this method and would tend to counteract the effects of fungal overgrowth. In the present experiments preservation of the explants after 20 hours, the maximum time for which the tissues were maintained in vitro, was generally good and certainly showed no greater degeneration than those explants inoculated by other means.

4.2.5 Conclusions

It would seem from the foregoing experiments that both the para-keratinized and non-keratinized rabbit oral mucosa are suitable tissues to use in an in vitro model of oral candidosis. The more rapid invasion of rabbit tongue mucosa as opposed to rat tongue may reflect a number of factors; *C. albicans* may be able to penetrate para-keratinized tissues more easily than ortho-keratinized epithelium, or the slightly higher inoculation dose used in the present experiments may influence the speed of invasion (see Chapter 5 for further discussion of this). However the different
<table>
<thead>
<tr>
<th>Infecting species</th>
<th>Rat tongue mucosa</th>
<th>Rabbit cheek mucosa</th>
<th>Rabbit tongue mucosa</th>
</tr>
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<tbody>
<tr>
<td>C. albicans</td>
<td>6</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>11</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>C. krusei</td>
<td>11</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>8</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>C. guilliermondii</td>
<td>8</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Controls</td>
<td>5</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

Inoculation dose: approximately 30 yeast cells

Incubation period: 30, 37, 40 & 47 hrs. for Rat tongue, 20, 30 & 40 hrs. for Rabbit cheek mucosa

Fixation: light microscopy for Rat tongue, scanning microscopy for Rabbit tongue

Incubation period: 12 & 15 hrs. for Rabbit tongue mucosa
strain of fungus used in the present experiments may also be more virulent and able to penetrate epithelium more rapidly than those used previously.

4.3 EXPERIMENT IV: THE INFECTION OF ORAL MUCOSA IN VITRO WITH SPECIES OF CANDIDA OF DIFFERING PATHOGENICITY

4.3.1 Materials and Methods

The preparation of explants from the dorsal tongue mucosa of rat and rabbit and the buccal mucosa of rabbit and their maintenance were carried out as described previously in experiments (1) and (III) respectively. Explants were inoculated after 4 hours or 20 hours with either 30 yeast cells of Candida as in experiment (1), or with a micro-colony as in experiment (III).

The fungal organisms used in these experiments were as follows: (see Appendix 1 for their source, classification, and maintenance).

<table>
<thead>
<tr>
<th>Species</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>3091A. 3118C, 'T'</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>3111</td>
</tr>
<tr>
<td>C. krusei</td>
<td>3100</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>3104</td>
</tr>
<tr>
<td>C. guillermondii</td>
<td>3090</td>
</tr>
</tbody>
</table>

The experimental protocol is set out in table 4.2.

4.3.1.1 Fixation and processing

Specimens for light microscopy were fixed and processed as in the previous experiments. Specimens for scanning microscopy were fixed in 1% glutaraldehyde in 0.02 M phosphate buffer for ¼ hour, then in 3% glutaraldehyde in the same buffer for 48 hours at room temperature. The tissues were then washed in distilled water for 1 hour, dehydrated in graded ethanols and using amylacetate as common solvent transferred to liquid CO₂. Critical point drying was carried out in a Polaron E 3000 apparatus (Polaron Equipment Ltd., Watford) by the method of Anderson (1951). The specimens were finally mounted on aluminum stubs using silver dag and sputter coated with gold in a Polaron Sputter coater E 5000 using a low pressure Argon atmosphere; the coating thickness was
Diagram 4.1. THE SPECTRUM OF INVASION OF CANDIDA SPECIES IN RAT TONGUE MUCOSA IN VITRO
(FUNGAL FORMS: Y-PHASE, M-PHASE, m = FEW SHORT HYPHAL FORMS)
between 20 and 50 nm. The specimens were viewed in a Cambridge Stereoscan 600 scanning microscope at an accelerating voltage of 15 KV and 25 KV.

4.3.2 Results

4.3.2.1 Histological assessment of keratinized explants

Maintenance of the uninfected control explants was satisfactory after all incubation periods, the structure of the keratinized epithelium and the connective tissues being similar to that seen in experiment (1).

In the infected culture systems Candida of all five species grew well and large numbers of fungi could usually be seen growing on and around the explants. Besides fungal growth on top of the keratin layers there was proliferation of organisms at the edges of the explant, where the connective tissues were exposed, providing the fungi with the opportunity of invading the connective tissue directly from the cut edges of the explants.

The general pattern of invasion by these species is graphically summarized in diagram 4.1. Explants inoculated with any of the strains of C.albicans showed a pattern of epithelial invasion from the surface similar to that seen in previous experiments. Penetration at the edges of the explant into the connective tissue was also rapid, the fungal hyphae growing upwards through the tissues so that by 37 hours, penetration of all the layers of the epithelium had also occurred. At 45 hours the explanted tissues were completely overrun by C.albicans.

In the culture systems infected with C.tropicalis both Y-phase and M-phase of the fungus were present. Heavy fungal growth took place on top of the epithelium but on no occasion did penetration of the keratinized layer and invasion of the deeper epithelial cell layers occur from this direction (fig. 4.5). However, proliferation of C.tropicalis at the edges of the explant occurred with striking M-phase invasion of the connective tissues. This enabled the fungus to penetrate the basement membrane and lower nucleated cells of the epithelium from below, although no penetration of the keratinized layers of the epithelium from this
Fig. 4.5  Dorsal mucosa of neonatal rat tongue infected with *C. tropicalis* and maintained for 30 hrs. Despite the large fungal colony on the surface of the tissues no invasion of the keratin layers can be seen. x 340 (p.a.S., haematoxylin).

Fig. 4.6  Neonatal rat tongue infected with *C. krusei*. The fungus is seen proliferating at the side of the explant, the M-forms are seen penetrating the connective tissue and basal epithelial cells; no penetration of the keratin layers is seen. x 140.

Fig. 4.7  A large colony of *C. guilliermondii* grows alongside an explant of rat tongue mucosa. No invasion of the tissues is seen. x 140.
direction was seen. Thus, by all appearances C. tropicalis lacked the ability to penetrate the intact keratinized layer either from above or below.

In the tissue infected with C. krusei the fungus grew in the Y-phase and M-phase but generally showed less invasiveness than C. tropicalis. Again, despite heavy growth of the fungi on the epithelial surface, no penetration of the keratinized layer was seen. Growth into the connective tissues at the explant edges was less extensive than with the previous two species, and only in a few explants did C. krusei reach and invade the nucleated cells of the epithelium (fig. 4.6).

Proliferation of C. parapsilosis and C. guilliermondii took place almost entirely in the Y-phase and only occasional short pseudohyphae were produced, mainly by C. parapsilosis. Active invasion of the epithelium or the connective tissue was not evident with either species (fig. 4.7). Despite heavy surface growth no penetration of the keratinized layer was seen, but there was sometimes flattening of the lingual papillae and atrophy of the epithelium beneath the surface colonies, similar to that seen with C. albicans (see fig. 5.5). Although only limited invasion of the connective tissue by these species occurred there seemed to be some degradation of the connective tissues in contact with colonies of yeasts at the explant edges (as in fig. 4.11).

4.3.2.2 Histological assessment of non-keratinized tissues

Histological examination of the non-infected control explants showed that the tissues in general had been adequately maintained. The appearance and structural organization of the non-keratinized mucosa was similar to that previously reported (section 4.2.3.1).

In the infected culture systems all species of Candida had proliferated readily. In those explants inoculated by means of a fine gauge wire, colonies of the fungi could be seen to grow on top of the epithelium, while those explants which had been inoculated with the suspension of yeasts showed proliferation of fungi mainly at the edges of the explants.
Diagram 4.2. THE SPECTRUM OF INVASION BY CANDIDA SPECIES IN RABBIT CHEEK MUCOSA IN VITRO (FUNGAL FORMS Y-PHASE, M-PHASE)
The pattern of invasion of the non-keratinized mucosa by the different species is summarized in diagram 4.2. Explants inoculated with any of the strains of *C. albicans* showed a pattern of invasion similar to that seen in experiment (III). Those explants maintained for long periods of time after infection, especially those inoculated with a micro-colony of yeast cells by wire inoculation, were very heavily overgrown by *C. albicans*.

In the culture systems infected with *C. tropicalis* Y-phase and M-phase of the fungus were present in colonies growing on the surface of the tissues. Invasion of the epithelium from the surface colonies was seen in explants maintained for 24 hours or longer, however the penetration by the hyphae of *C. tropicalis* did not extend beyond the spinous layer of the epithelium even in explants maintained for 30-40 hours after inoculation (fig. 4.8). In those explants inoculated with the liquid suspension hyphae proliferated at the explant edges, and invaded adjacent connective tissues, passing through the basement membrane to enter the deeper cell layers of the epithelium.

*C. krusei* also penetrated the surface of the non-keratinized tissues and invaded the superficial epithelial layer but hyphae had only penetrated to the upper spinous layers by 40 hours after inoculation (fig. 4.9). Growth of the fungus through the connective tissues, from the explant edges was also apparent (fig. 4.10).

Heavy surface proliferation of *C. parapsilosis* and *C. guilliermondii* took place almost entirely in the Y-phase and as with the infected keratinized explants no invasion of either epithelium or connective tissue occurred (fig. 4.11).

4.3.2.3 Assessment of infected tissues by scanning electron microscopy

a) The epithelium

The surface of the rabbit tongue mucosa showed closely packed bulbous filiform papillae on which the outlines of the superficial squames could be clearly seen. These surface cells had a hexagonal shape and at
Fig. 4.8  Rabbit cheek mucosa infected with C. tropicalis by means of wire and maintained in vitro for 30 hrs. The M-form can be seen invading the superficial epithelial cell layers. x 400.

Fig. 4.9  C. krusei proliferating in amongst the superficial cell layers of non-keratinized mucosa after 30 hrs. in vitro. It appeared that invasion of epithelial cells did occur. x 400.
**Fig. 4.10** *C. krusei* proliferating at the side of an explant. Hyphal forms are seen invading through the connective tissue into the deep epithelial cell layers. x 425.

**Fig. 4.11** A colony of *C. parapsilosis* spreads over the non-keratinized epithelium and adjacent connective tissue. No invasion of epithelium is seen, although some degeneration of the connective tissue in contact with the yeast cells appears to be occurring. x 250.
Fig. 4.12 Scanning micrograph of rat tongue keratinized mucosa invaded by C. albicans. (B=blastospore, H=hypha, S=bud scar). x 32,000.

Fig. 4.13 Scanning micrograph of the area of invasion of a keratin squame by a hypha of C. albicans. (D=irregular masses of surface material). x 120,000.
higher magnification each showed a finely ridged and pitted surface pattern (see fig. 4.13).

Rabbit cheek mucosa presented a much smoother surface, but still showed the outlines of the large surface squames, some of which seemed so loosely attached to the underlying cells that they appeared to be desquamating. At higher magnification the surface had a corrugated appearance but this was less marked than in the rabbit tongue mucosa (see fig. 4.14).

b) The fungus

In those explants infected with *C. albicans* both blastospores and hyphae of the organism were clearly visible on the surface of the tissues, and in many cases hyphae could be seen extending from the parent blastospore as germ tube formation proceeded (fig. 4.12). The blastospores themselves were generally rounded or slightly ovoid in shape and about 3-4μ in diameter. Their surface seemed fairly smooth, occasional truncated bud scars being visible where budding yeasts had broken away from each other, where buds and parent cells remained attached a deep groove separated the individual cells. The hyphae, about 1μ in diameter, usually extended from blastospores and often showed a less smooth appearance due to irregular masses of surface coating. Similar material was often deposited over the surface of the other *Candida* species and the epithelial squames, however it was not possible to determine whether this material originated from the *Candida*, the oral tissues or merely represents some other form of extraneous deposit (fig. 4.13).

The Y-phase and M-phase of *C. tropicalis* had a similar appearance to *C. albicans* (fig. 4.14), while the rounded blastospores of *C. krusei* were generally smaller than those of the two previous species, but the majority of *C. krusei* cells present were elongated and up to 8μ in length (fig. 4.15). The individual cells seemed to be joined together with constrictions between adjoining cells; presumably these represented a pseudomycelial form. The surface characteristics of the cells were similar to the other species. *C. parapsilosis* and *C. guilliermondii* were
Fig. 4.14  Micrograph of *C. tropicalis* growing on the surface of non-keratinized rabbit cheek mucosa. The hyphae appear to be invading the cells (†). x 25,000.

Fig. 4.15  Micrograph showing possible areas of invasion of non-keratinized mucosa by *C. krusei* (‡). x 16,000.

Fig. 4.16  Y-forms of *C. parapsilosis* proliferating on the surface of cheek mucosa. No disturbance of the tissue in contact with the yeasts was seen. x 20,000.
present almost entirely in the Y-phase, round or ovoid blastospores of various sizes. *C. parapsilosis* however did have some short pseudohyphal forms represented by one or two elongated cells separated by deep constrictions. Bud scars were regularly present where cells had separated from each other (fig. 4.16).

**c) Invasive processes**

Considering the invasive activity of the species in the infected explants, examination of the specimens in the scanning electron microscope confirmed the results of light microscopy. In the keratinized explants infected with *C. albicans*, the hyphae could be seen invading the superficial epithelial cells (fig. 4.13). However in the keratinized explants infected with other species of *Candida* no evidence of invasion of the superficial epithelium by the fungi was seen, even though the latter proliferated in close association with the surface cells. In the non-keratinized mucosa explants evidence of the invasion of *C. albicans*, *C. tropicalis* and possibly *C. krusei* was seen (fig. 4.15). A detailed account of the relationship between *C. albicans* and the epithelium will be given later (see Chapter 6).

**4.3.3 Discussion**

The five species of *Candida* used in these experiments showed a gradation of invasive activities in oral mucosa in vitro, reflecting the pathogenicity of the fungal species demonstrated in living animals (Hurley, 1966) and in cell culture systems (Hurley & Stanley, 1969). *C. albicans* was undoubtedly the most invasive, being able to penetrate all layers of the epithelium and connective tissue in both keratinized and non-keratinized mucosa.

Three strains of *C. albicans* were used in the model system and the patterns of invasion of each strain in the various mucosa were similar. The slight differences in the rate of penetration of the epithelial tissues by the strains might be due to experimental variability or be attributable to differences in the virulence of the fungi which have been
detected between the isolates of \textit{C. albicans} \cite{partridge1971, wain1976}.

The species of \textit{Candida} used in these studies have all been isolated at one time or another from human sources either as pathogens or commensals and have been shown to cause systemic candidosis. Histological studies on infected cell cultures have shown that hyphae of other \textit{Candida} species besides \textit{C. albicans} are able to penetrate nucleated epithelial cells \cite{hurley1969} and this was supported in the present experiments. \textit{C. tropicalis} and \textit{C. krusei} invaded the deeper nucleated cells of epithelium and the superficial layer of non-keratinized mucosa even though they were unable to penetrate superficial keratinized tissues. The ability of \textit{C. tropicalis} and \textit{C. krusei} to invade the non-keratinized mucosa \textit{in vitro} from the surface tends to support the hypothesis that these species are able to invade mucosa \textit{in vivo} and they have in fact been isolated from oral and vaginal candidosis \cite{hurley1966}. The isolation of \textit{C. parapsilosis} and \textit{C. guilliermondii} from superficial lesions of candidosis is a rare occurrence. In a three year period, pure cultures of these organisms were only isolated each from one undoubted lesion of vaginal thrush \cite{hurley1966}. In the present studies only the strain of \textit{C. parapsilosis} used produced short pseudohyphae, \textit{C. guilliermondii} remaining entirely in the yeast phase, and there was no evidence that the species were capable of invading oral epithelium.

Hurley and Stanley \cite{hurley1969} proposed that specific aggressive mechanisms are possessed to a high degree by M-phase producing species such as \textit{C. albicans} and \textit{C. tropicalis} and only to a low degree by species such as \textit{C. guilliermondii} in which the Y-phase predominated. This hypothesis is supported in the present studies as only \textit{C. albicans} was able to penetrate the barrier of the keratin layers in orthokeratinized mucosa and invade the rest of the epithelium.

The failure of \textit{C. tropicalis} and \textit{C. krusei} to penetrate the keratin layer of rat tongue mucosa is not indicative of a general inability to invade cells but suggests that the keratinized squames offer particular
resistance to less pathogenic species. It seems however that this is not true of all keratinized epithelia for in experimental cutaneous candidosis in rodents Ray and Weupper (1976a) have recently described how hyphae of non-pathogenic species of Candida (C.tropicalis, C.krusei, C.parapsilosis and C.guilliermondii were among those used) were able to penetrate the keratin layer within 24 hours of infection. However unlike the pathogenic species, C.albicans, the non-pathogens failed to penetrate the malpighian layers and form lesions. It thus seems that even if C.tropicalis and C.krusei were able to penetrate non-keratinized oral mucosa in vivo, and the present work suggests that they are able to in vitro, lesion formation may not inevitably occur. However the possibility remains that the more virulent strains of the hyphal producing species including C.tropicalis and C.krusei, as well as invading are able to initiate lesion formation, and this would account for the reports in the literature of their being isolated from mucosal lesions.

Apart from the invasion of tissues, other pathogenic effects of the fungi on the cultured tissues, such as the atrophic changes in epithelium associated with heavy surface proliferation of fungi, were common to all the fungal species. Such changes might be attributed to the action of fungal metabolites within the culture system and will be discussed further with particular regard to C.albicans in Chapter 5.

The pattern of progressive invasion of oral epithelium by the species of Candida in the model system reflecting their differing pathogenicity, seems to indicate that the mucosal tissues in vitro retain their structural integrity, so as to offer resistance to invasion by all but the most pathogenic fungi. In other words, the mucosa in vitro is not merely an accumulation of cells serving as a passive growth medium for the fungi irrespective of their pathogenicity, but behaves in many ways like tissue in vivo.

4.4 CONCLUSIONS

The series of experiments described in this chapter represent an evaluation of the model system of oral candidal infection. It has been
established firstly, that other types of mucosa besides keratinized rat tongue can be used in the *in vitro* system, so increasing its usefulness. Secondly that the pattern of invasion of the host tissues by a number of strains of *C. albicans* was consistant, and thirdly that the integrity of the mucosal tissues is retained *in vitro* and the epithelial behavior associated with fungal infection is comparable in many ways with that *in vivo*. These findings support the *in vitro* model of candidal infection of the oral mucosa as a useful experimental system in which to investigate in detail the relationship established between invading fungi and host epithelium.
CHAPTER 5

THE HISTOLOGICAL APPEARANCE OF CANDIDAL INFECTION OF ORAL MUCOSA IN VITRO
In the previous two chapters a method has been described for the production of candidosis in oral epithelium in vitro. This chapter discusses the histological features of neonatal rat tongue and neonatal rabbit tongue and cheek infected with Candida. While differences exist between the histopathological pictures presented by the in vivo and in vitro infections, the relationship established between the fungus and epithelium in vitro does resemble that previously reported in infections of rat mucosa in vivo (Jones & Adams, 1970; Jones & Russell, 1973(a)) and there are many features similar to those described in human candidosis (Cawson, 1966).

Careful examination of serial sections of the infected explants obtained from the previous experiments revealed certain characteristic features in the pattern of fungal invasion in vitro with respect to the growth and proliferation of C. albicans, the fungal invasion of the mucosa and the effect of such fungal colonization and invasion on the explanted tissues. It is proposed to discuss these features in more detail in the present chapter.

In the review of the literature, it was pointed out that a number of factors associated with both the host and the fungus appear to influence the colonization and invasion of an epithelium by C. albicans in vivo (see sections 2.5, 2.6). In the in vitro system some factors, such as the saliva and the competitive oral flora, are absent and play no part in regulation of fungal growth. However, other factors are retained; the experiments of Chapter 4 suggest that the epithelial tissue maintains its structural integrity and barrier function in vitro. Of the factors associated with the fungus, both the number of organisms and the form in which C. albicans is present would appear to be influential. In the present studies, however, no pattern emerged with regard to the numbers of fungi present. In some cases heavy surface colonization of the explants by C. albicans was associated with minimal invasion of the tissues, while in other cases light surface proliferation of the fungi was associated with deep invasion. Indeed, a single hypha
**Fig. 5.1** *C. albicans* proliferating in the duct of a minor gland in cheek mucosa, and invading the adjacent tissues. x 157.

**Fig. 5.2** Invasion of non-keratinized epithelium by *C. albicans*. Long hyphae migrate downwards through the tissue. x 500.

**Fig. 5.3** *C. albicans* invading tissues from the edge of a rat tongue explant. Long hyphae travel up towards the epithelium. x 280.
seemed capable of invading the underlying epithelial cells and Wain et al. (1976), studying candidal infection of chick chorio-allantoic membrane, also described invasion of epithelium by isolated fungal cells. The appearance of the M-phase of C. albicans has always been regarded as significant, indicating tissue invasion in vivo (Mackenzie, 1964). However the growth of C. albicans in tissue culture media takes place in both the Y-phase and M-phase and thus the development of hyphae in vitro may not be so important. Nevertheless, it did seem that the M-phase of C. albicans was more often present at the site of invasion of the epithelial surface and in close approximation to the superficial squames. Host cell exudates and cell constituents have both been reported as influencing fungal form in vivo (Higgs, 1973b; Barlow et al., 1974) and it may be suggested that such factors, present in the microenvironment of the epithelial surface squames in vitro, encourage the formation of germ tubes and subsequent invasion of the host tissues by the M-phase. However, it remains difficult to define the factors involved in the initiation of invasion in the present model system.

A further influence on the invasive pattern would seem to be the topography of the tissue surface. In the tongue mucosa of both rat and rabbit accumulation of the fungi tended to take place in the inter-papillary regions, whereas in the non-keratinized mucosa the fungi proliferated readily in the surface openings of the ducts of the minor salivary glands present in the cheek mucosa (fig. 5.1). While this may reflect gravitational flow of the fungal cells on the tissue surface these areas seemed to provide the right microenvironment for proliferation and invasion by C. albicans. Ray and Weupper (1976(a)) in studies of experimental cutaneous candidosis in rodents commented on the predominant peri-follicular involvement and suggested that it may reflect the ease of cell penetration at such sites, due possibly to the moisture and hydration of the epidermis in these areas. Similar factors may influence the behavior of Candida in the peri-ductal regions of non-keratinized mucosa in vitro. In the case of rat tongue explants the epithelium of the inter-papillary regions and the anterior surface
of the filiform papillae consists of a soft-type of keratinization (Farbman, 1970) which C. albicans may find more amenable to invasion than the hard-type keratin seen in the posterior aspect of the papillae.

The M-phase of C. albicans penetrates the superficial epithelial cell layers by an unknown mechanism to become an intracellular parasite and is the predominant phase present in the epithelium in vitro. Where the superficial squames of the epithelium appeared to be loosely attached intercellular budding of yeasts could be seen. In the deeper epithelial layers the M-phase was frequently seen to undergo lateral budding. However in no case was the distribution of morphological forms as clear cut as that described by Taschdjian et al. (1960) in experimental infection of mouse vagina in which the Y-phase predominated in the superficial layers of epithelium and the M-phase in the deeper layers.

No distinctive pattern of growth of the hyphae through the epithelium could be seen, for although the predominant direction of growth was down through the epithelium many hyphae seemed to grow horizontally through epithelial cells. The vertical migration through the epithelial cell layers was most apparent in the non-keratinized mucosa (fig. 5.2). Cawson has commented on the appearance of long straight hyphae in human mucosa in vivo (1966). By comparison, growth of the fungi through the connective tissue and into the deeper epithelial cell layers from below, which follows fungal proliferation at the sides of the explant, was associated with long hyphae travelling up through the tissues as if attracted by the epithelium (fig. 5.3).

The speed of hyphal penetration of the tissues was remarkably rapid. There was an initial lag phase before the invasion took place, but once the hyphae had penetrated the superficial epithelial cell layers, invasion of the deeper layers followed in a matter of hours. By means of a micrometer eyepiece the rate of penetration of the hyphae into the histological specimens was assessed. Assuming a minimum of a two hour lag phase before germ tube formation and subsequent tissue invasion occurred, a conservative estimate of the elongation rate in epithelium was 17 μ/hour (0.3 μ/min). Growth through the connective
Fig. 5.4  Hyphae of \textit{C. albicans} penetrating non-keratinized mucosa and causing distortion of the epithelial cell membranes (†). x 440.

Fig. 5.5  \(\text{Y-forms of }\textit{Candida}\) proliferating on the surface of rat tongue, associated with flattening of the lingual papillae after 39 hrs. in culture. x 350.
tissue seemed faster, due presumably to lack of resistance, but the
tortuous path of penetration made it less easy to obtain a reliable
estimate. The measured rate seemed comparable with the growth rates
of 0.45 µ/min and 0.37 µ/min reported in the literature for *C. albicans*
in human serum or plasma (Davies & Denning, 1972; Wain et al., 1976).

Given such rapid and extensive penetration of tissues by *C. albicans*
the effects on the epithelial cells in vitro seen in the light microscope
were minimal. After moderate invasion of the tissues signs of epithelial
degeneration were rarely seen even at 40 hours post infection and, as
with in vivo infection, the individual epithelial cells parasitised by
*Candida* rarely showed significant changes even when the fungi had
apparently penetrated the nucleus of the cell. However the epithelial
cells in heavily invaded areas did sometimes show nuclear pyknosis and
lack of cytoplasmic staining indicative of cell degeneration. Penetration
of the non-keratinized epithelial cells, especially of the superficial
cell layers, was often accompanied by marked distortion of the epithelial
cell membranes whether invaded from above or below (see fig. 5.4). Such
distortion of the epithelial cell membrane was not so frequently observed
in keratinized tissue. Presumably the plasma membrane of the non-
keratinized tissues, which becomes thickened during maturation (see
section 2.3) offers more resistance to penetration than the interior of
the cell which remains hydrated (Silverman, 1971) so results in the
membrane distortion. In comparison the rigid keratin squames filled with
tonofilaments seem to offer uniform resistance to fungal penetration.
Whatever the explanation the appearance in the non-keratinized tissues
is one of *C. albicans* pushing its way through the cells which would imply
some mechanical disruption of cell membranes during the invasive process
and discount an entirely enzymatic process.

The effects of extensive proliferation of fungi on the maintenance
of the epithelium was somewhat variable. In some cases large colonies
of *Candida* spread over the surface without causing any apparent changes
in the epithelial maintenance, while in others alterations were apparent.
Flattening of the filiform papillae (fig. 5.5) and atrophic changes in
the epithelium represented by a reduction in the number of epithelial cell layers was occasionally seen to be associated with heavy surface growth of all the Candida species used in the experiments of Chapter 4 including C. albicans. Such pathogenic effects might be attributed to the action of fungal metabolites within the culture system altering the maintenance of the tissues, but Jones and Russell (1973a) observed flattening of the lingual papillae in experimental infection of rat mucosa in vivo while Buctz-Jørgensen (1974) described atrophic changes of palatal epithelium in experimental candidal stomatitis in monkey, even though no intraepithelial fungal penetration was found. This suggests a direct effect of the fungi upon the host epithelial cells.
CHAPTER 6

THE ULTRASTRUCTURE OF THE
HOST-PARASITE RELATIONSHIP

6.1 INTRODUCTION

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6.3.2.3 The fine structure of the host-parasite relationship in vivo

6.4 DISCUSSION

6.5 CONCLUSIONS
6.1 INTRODUCTION

One of the foremost difficulties encountered in examining the fine structure of the relationship established between parasitic fungi and host tissue is that of obtaining adequate fixation of the very different tissues involved. The fixation of yeasts has presented severe problems to electron microscopists and prolonged fixation with potassium permanganate alone (Luft, 1956; Atzelius, 1962) or in combination with other fixatives (Montes et al., 1965; Iwata, et al., 1973) has been most frequently used. More recently successful elucidation of the cell wall structure of C. albicans has been achieved using acrolein, Tapo and osmium (Djaczenko and Cassone, 1972; Cassone et al., 1973) while the fine structure of the internal organelles of C. albicans has been demonstrated by Borgers and DeNollin (1974) by glutaraldehyde and osmium fixation of cryostat sections of the yeasts.

Methods of fixation similar to those which have proved adequate in the examination of yeast cells alone have also been used successfully to examine parasitized plant tissues (Hess, 1966), while Montes and Wilborn (1968) used acrolein and osmium in the first ultrastructural investigations of superficial candidosis. However aldehyde and osmium are usually the fixatives of choice for oral mucosa and osmium fixation alone has proved successful in the examination of mucosal candidosis (Cawson & Rajasingham, 1972; Mohammed, 1975). In the following studies a number of fixation regimes were employed using both aldehydes and osmium as primary fixatives.

6.2 MATERIALS AND METHODS

Tissues for the ultrastructural studies consisted of:
1) Uninfected, control explants of keratinized rat tongue mucosa, para-keratinized rabbit tongue mucosa and non-keratinized rabbit cheek mucosa maintained in vitro for up to 36 hours.
2) Explants of each of the above tissues infected with strains of *C. albicans* (see Appendix I) as in Chapter 1 and 4, and maintained in vitro for 15-30 hours.

3) *In vivo* material: a gingival biopsy from a patient with chronic muco-cutaneous candidosis attending the Oral Medicine Clinic at the London Hospital Medical College.

The tissues were harvested and fixed by the following methods:

- a) Palade's veronal acetate buffered osmium tetroxide (1%) pH 7.3-7.5 (Palade, 1952).
- b) s-collidine buffered osmium tetroxide (1.33%) at pH 7.45 (Bennet and Luft, 1959).
- c) s-collidine buffered glutaraldehyde (3%) at pH 6.8 followed by osmium tetroxide (1%) at pH 7.4 (Garrison and Boyd, 1975).
- d) phosphate buffered glutaraldehyde (2.5%) at pH 7.3-7.5 followed by osmium tetroxide (2%) (Rooney and Moens, 1973).
- e) phosphate buffered 2% formaldehyde and 2.5% glutaraldehyde (modified: Karnovsky, 1965) followed by Millonig's osmium tetroxide pH 7.3 (Millonig, 1961).

### 6.2.1 Processing

After washing in distilled water the tissues were rapidly dehydrated in ascending concentrations of ethanol and transferred to dry acetone at room temperature. Specimens remained for a period of about 24 hours in a mixture of dry acetone and Epon 812 (Luft, 1961) and were finally flat embedded in Epon 812 before being polymerized at 80°C. for 10 minutes, 47°C, for 2 days, and 60°C. for 2 days.

### 6.2.2 Preparation of Specimens for Ultrastructural Examination

Sections with silver-gold interference colours were cut on a Huxley ultramicrotome using glass or diamond knives, and collected on either single holed or 200-mesh copper grids which had been thinly coated with formvar (polyvinyl formal formvar). The sections were then stained with 0.5% aqueous uranyl acetate at 60°C. for 5 minutes and lead citrate after
Reynolds (1963), and examined in either an A.E.1 EM6B or Siemens 101 electron microscope at an accelerating voltage of 60 kV.

6.2.3 Examination of Infected Explants in the Scanning Electron Microscope

As part of the investigation into the mechanism of invasion of epithelium by \textit{C. albicans} some infected explants were examined in the scanning electron microscope. The specimens were those prepared for the studies in Chapter 4 and consisted of rabbit cheek and tongue mucosa maintained for 15 hours after infection with \textit{Candida} (see section 4.3.1).

6.3 RESULTS

6.3.1 Ultrastructural appearance of uninfected control tissues

6.3.1.1 Rat tongue

Examination of uninfected explants of the dorsal mucosa of rat tongue maintained \textit{in vitro} for up to 35 hours revealed a relatively normal appearance. Some variability in the maintenance of epithelial organization could be seen in different specimens, while tissues at the edges of explants had undergone some disorganization. However in the majority of specimens the undulating basal lamina was of uniform density and exhibited a granular-fibrillar structure; hemi-desmosomes were numerous and situated at regular intervals along the lamina. The basal epithelial cells were columnar with an oval nucleus in which chromatin stippling was often seen. The spinous cells appeared normal with distinct nuclei and numerous desmosomal attachments. Intercellular spaces between these cells were minimal although this appearance depended on the method of fixation. Tonofilaments, mitochondria and ribosomes were clearly visible and generally of normal appearance in these cells. Occasionally some swelling of the mitochondrial membranes was seen in cells which appeared to be more densely stained.

Considerable amounts of glycogen were present throughout the epithelial cell layers especially in the upper spinous and granular layers and is probably a feature of neonatal tissues (McFall & Kraus, 1963). There appeared to be no change in the amount of glycogen evident throughout the period of culture.
As mentioned in the histological description, the rat tongue undergoes a dual pattern of keratinization (Farbman, 1970). At the ultrastructural level the existence of a population of cells producing hard keratin and one producing soft keratin was clearly visible. Keratohyalin granules of a heterogenous type were present only in the regions of soft keratin; that is the anterior surface of the papillae and interpapillary regions, where the granular cells also contained scattered tonofilaments and many lamellated membrane granules. In this region the change from the granular layer to the keratin layer is abrupt. On the posterior aspect of the papillae, epithelium producing hard keratin could be seen. No distinct granular layer was present, and the upper spinous cells changed abruptly to darker staining cells packed with bundles of tonofilaments.

As in vivo, the most superficial squames generally appeared less dense than those beneath them due to rehydration of the cells by surface moisture. However these squames also appeared to be detached from each other and probably represent cells which would have been desquamated in vivo.

Tissues underlying the epithelium showed a greater disorganization. Early cellular degeneration was frequently seen, including swelling of mitochondria and the presence of large densely staining intracellular bodies, probably lipid inclusions but other connective tissue cells were of normal appearance.

6.3.1.2 Rabbit tongue

The fine structure of the dorsal mucosa of rabbit tongue was well maintained in vitro throughout the maximum period of incubation of 20 hours. Changes in the structure of both epithelium and connective tissues were similar to those seen in the rat mucosa in vitro but generally of a lesser degree due to the shorter period of in vitro maintenance. The specimens examined were predominantly para-keratinized, vestiges of pyknotic nuclei and other organelles being present in the superficial keratinized squames, although a few specimens showed a more fully ortho-
keratinized epithelium. No keratohyalin granules were present in the mid epithelial layers but the organization of the deeper layers i.e. basal and spinous was similar to that of rat tongue.

6.3.1.3 Rabbit cheek

Maintenance of rabbit buccal mucosa in vitro for up to 20 hours appeared satisfactory at the electron microscope level. Some disorganization of the connective tissues could be seen and was similar to that observed in tongue explants described above. The non-keratinized mucosa in vitro showed a continuous basal lamina beneath the cuboidal basal cells. These cells contained round or oval nuclei, many free ribosomes, and occasional tonofilaments. An increase in cell size from lower to upper spinous cell layers was associated with a decrease in the concentration of tonofilaments, ribosomes, mitochondria and other cell organelles; membrane coating granules with a dense central core could be seen at the superficial border of the upper spinous cell layer. Desmosomal attachments between all cells appeared normal. A typical granular cell layer is absent from non-keratinized epithelium, but the deeper cells of the superficial third of the buccal epithelium contained occasional uniformly rounded, dense keratohyalin granules; these were associated with ribosomes but not tonofilaments.

In the superficial cell layers the nuclei varied from appearing plump with regular and irregular chromatin stippling, to appearing shrunken and often pyknotic. The tonofilaments appeared sparse and short and in most of the superficial cells the cytoplasmic organelles were virtually absent, only a few mitochondria and small complexes of endoplasmic reticulum and ribosomes being seen. Some cells showed a greater density of filaments, a complete absence of organelles and appeared to be more fully keratinized.

Large amounts of glycogen were present in many of the cells of the non-keratinized mucosa, although in some areas it appeared that preservation of this substance had been incomplete and it had been lost, leaving clear areas in the cell cytoplasm.
**Fig. 6.1** Scanning micrograph of a hypha of *C. albicans* penetrating a keratinized squame of rat tongue mucosa. Little disturbance of the cell membrane is seen at the point of penetration. x 70,000.

**Fig. 6.2** Transmission micrograph showing hyphae of *C. albicans* invading rabbit tongue mucosa. The fungi penetrate intracellularly and a close seal is established between the epithelial cell and fungal cell wall. (Short term glutaraldehyde and osmium fixation). x 4750.
6.3.2 Ultrastructure of the Infected Explants

Examination of infected explants in both scanning and transmission modes confirmed that *C. albicans* had invaded both epithelial and connective tissues, and in many cases had become an intracellular parasite. In the transmission studies the overall pattern of candidal infection of the tissues was similar in all the methods of fixation employed, the only differences being in the details of appearance. Therefore, it is not proposed to give a detailed account of each fixative, but to refer only to the significant features of the fixation methods in the descriptions that follow.

6.3.2.1 The fine structure of the host-parasite relationship seen in the scanning microscope

In Chapter 4 an account was presented of the ultrastructure of *C. albicans* and its invasion of keratinized and non-keratinized mucosa seen in the scanning microscope. At this point it remains to emphasize the details of the relationship between the host and parasite during invasion. In the scanning microscope little disturbance of the epithelial cell surface at the site of fungal penetration was apparent, except for some loss of the surface ridging of the squames. Similarly there were no changes in the surface structure of the hyphae in the vicinity of penetration. They appeared to merge with the epithelial cells, and on occasion were seen to pass right through the surface squames, the only visible change between the two being an alteration in the emission intensity between the hypha and epithelial cell surface (fig. 6.1).

6.3.2.2 The fine structure of the host-parasite relationship seen in the transmission electron microscope

Although both yeast and hyphal forms of *C. albicans* were probably present on the surface of the explants the sections examined revealed a fairly uniform population of fungal transections so that except when a hypha was cut in a longitudinal direction, it was not possible to make any distinction between the fungal forms on the basis of profile (fig. 6.2 & 6.3). It has been suggested that the thickness of the cell wall differs
Fig. 6.3 Transmission electron micrograph showing *C. albicans* invading rabbit tongue mucosa. Little disturbance of the host cells by the fungi is seen. There appears to be marked variation in the organization of the fungal cell wall. (prolonged glutaraldehyde & osmium fixation). x 6240.
Fig. 6.4 The cell wall of *C. albicans* is clearly demonstrated by glutaraldehyde and osmium fixation. x 36,000. The inset shows that five layers can be distinguished in the cell wall. x 90,000.

Fig. 6.5 Changes can be seen in the organization of the cell wall during budding (↑). x 17,500. (Short-term glutaraldehyde & osmium).
in the two forms; Cassone et al. (1973) reported the average thickness of the germ tube wall to be half that of the blastospore. Some variation in the cell wall thickness of the fungi within the tissues could be observed in the present studies however it was not possible to correlate this with fungal form, or maturation of the hyphae. Where longitudinal sections of hyphae were present, cross walls were often seen.

Using osmium alone as a fixative, the cell wall appeared as an almost homogenous structure of moderate electron density with a dense exterior margin and a thin dense inner layer adjacent to the plasma membrane of the yeast cell. However, prefixation with glutaraldehyde often gave clear definition of the cell wall; the methods of Rooney and Moens (1971) or of Garrison and Boyd (1975) enabled five layers within the cell wall to be distinguished (fig. 6.4). The exterior layer was a flocculent layer, particularly pronounced in the prolonged fixation (Garrison & Boyd) and in budding hyphae in the shorter fixation (Rooney & Moens) (fig. 6.5). The amount of flocculent material varied considerably from fungus to fungus and also appeared to be related to the activity of the fungus. When C. albicans was located extracellularly amongst the surface squames the layer was very distinct, but it became indistinguishable when the fungi were in close contact with processes of the epithelial cells (fig. 6.6(a)). As invasion of the cell took place however the flocculent material reappeared (fig. 6.6(b)). Fungi within cells did, however, show variation in the amount and density of outer cell wall layer (fig. 6.3). On some occasions it extended into the surrounding cell cytoplasm to a considerable distance (fig. 6.6(c)) and even seemed capable of spreading to adjoining epithelial cells (fig. 6.6(d)).

The second layer was formed by a highly dense material and was the most electron opaque layer, while the third consisted of filaments which formed bundles orientated parallel to the cell surface (fig. 6.4). The fourth layer was electron lucent and contained dispersed material of moderate electron density. The third and fourth layers were often not well demarkated. The final layer was closely opposed to the plasma membrane, and was narrow and of high electron density.
Fig. 6.6 These micrographs show the variation in the appearance of the outer cell wall of the fungi during invasion. A thick outer layer is seen in fungi among the surface squames (a) and processes of the epithelial cells appear to be closely associated with it (b). The layer disappears when in contact with the epithelial cell membrane (b), but reappears when the fungus becomes intracellular (c), often extending some distance into the host cell cytoplasm and appears capable of spreading into adjacent cells (d) (Prolonged glutaraldehyde & osmium). (a) x 22,500; (b) x 17,500; (c) x 15,300; (d) x 12,600.
Fig. 6.7  Clear definition of intracytoplasmic detail is seen in these intraepithelial hyphae after 39 hrs. in vitro. Many ribosomes are present in the cytoplasm. M=mitochondria, C=cytoplasm, PM=plasma membrane. (Palade's osmium) x 22,100.

Fig. 6.8  The fungal cell cytoplasm maintained an electron dense appearance when fixed with glutaraldehyde although occasionally, as here, organelles could be distinguished. N=nucleus. x 29,700.
Structural changes in the cell walls were clearly observed in hyphae which appeared to be budding and seemed to involve all the layers (fig. 6.5). At the site of germination the majority of the cell wall appeared to consist of a proliferating layer full of medium dense material similar to that of the fourth layer of the mature hyphal wall. The fibrous third layer was absent while the outer layers were less clearly defined.

An electron dense plasma membrane was seen bordering the inner surface of the cell wall. It was most clearly defined when specimens had been fixed with osmium alone (fig. 6.7). This tri-laminar membrane generally showed many short invaginations and seemed to have the micro-villous appearance reported by Cawson and Rajasingham (1972). Breaks in the membrane and continuity with internal membrane structures were occasionally seen.

The contrast of the internal organelles and cytoplasmic membrane structures in C. albicans was generally low, the fungal contents generally having a uniformly dense appearance, and only faint outlines of intracytoplasmic detail being apparent. Osmium tetroxide fixation alone did allow better delineation of internal structures, although this varied considerably from specimen to specimen. Palade's osmium provided the best definition of internal membranes and it was generally the intra-epithelial hyphae which had the most clearly defined organelles (fig. 6.7).

Fixation with aldehydes increased the electron density of all the cell contents to a degree which often prevented any definition of internal structure except where fixation had been fairly short, as in the method of Rooney and Moens (fig. 6.8). Nuclei were only rarely observed in the fungal cells. Numerous ribosomes packed the cytoplasm either singly or in polysomal arrays and seemed to be partly responsible for the dense appearance of the cellular contents. The most obvious organelles were mitochondria with well developed cristae, randomly distributed in the fungal cytoplasm. A vacuolar apparatus lined by smooth membrane and containing electron lucent amorphous material often occupied a central position in the cytoplasm of hyphae. Short fragments of smooth membranes, vesicles and lipid-like droplets were also visible.
**Fig. 6.9** A micrograph showing a hypha penetrating through a keratin squame. Minimal disturbance of the host cell membrane and cytoplasm is seen; tonofilaments abut directly onto the fungal cell wall. x 29,700.

Inset shows the close approximation of the epithelial cell membrane to the hypha. x 88,000. (short-term glutaraldehyde & osmium).
In the infected tissues, hyphae of *C. albicans* had penetrated both between and into the epithelial and connective tissue cells. A similar pattern of invasion was seen in the keratinized and non-keratinized epithelium. Many fungi clustered between the loosely attached superficial squames although hyphae could also be seen extracellularly in the deeper layers in close association with the cell membranes of the spinous and basal cells. Hyphae of *C. albicans* were frequently seen entering a cell or passing from one epithelial cell to another, this provided the opportunity of examining the relationship between the host cells and the fungi during penetration of the epithelial cell. Minimal disturbance of the host cell membrane took place and the plasma membrane of the epithelial cell was tightly opposed to the fungal cell wall in these circumstances (fig. 6.9). Only on one occasion was any discontinuity of a cell membrane seen in association with fungal invasion, in all other cases no breakdown of the epithelial cell membrane was seen unless it was in direct contact with the fungal cell wall. The distortion of the non-keratinized cell membranes evident in the light microscope was also seen in the electron microscope and in one instance a hypha penetrating an epithelial cell nucleus was seen to cause considerable distortion of the nuclear membrane (fig. 6.10).

Within the epithelial cells the fungi lay free in the cytoplasm and in the superficial squames appeared to cause little disturbance. In many cases tonofilaments and other cytoplasmic contents abutted directly against the fungal cell wall although some of the hyphae were seen to be surrounded by a electron lucent space of variable size (fig. 6.11) which occasionally contained material that appeared to be derived either from the fungal cell wall or the epithelial cell cytoplasm. In explants in which superficial cells contained much glycogen it seemed that the fungi had a predilection for such cells, although no obvious depletion in the glycogen content was observed. There was a similar lack of intracellular disturbance when *C. albicans* became an intracellular parasite of the spinous and basal cells, the nuclei, mitochondria and other epithelial cell organelles were generally of normal appearance and showed no degenerative changes. However some cells containing hyphae did have an
Fig. 6.10  This micrograph shows a hypha penetrating a nucleus of a cell from the spinous layers of non-keratinized epithelium. Marked distortion of the nuclear membrane appears to be caused by the fungus which appears to be surrounded by a number of membranes besides the distorted nuclear membrane. (Short-term glutaraldehyde & osmium). x 18,500.
Fig. 6.11 While many sections of intracellular fungi showed close contact between the epithelial cell cytoplasm and fungal cell wall, some showed a loss of cytoplasm around individual fungal profiles (>). This was suggestive of enzymatic digestion of the host cell cytoplasm by the advancing hyphae. (Short-term glutaraldehyde & osmium). (a) x 33,000; (b) x 15,000.
Fig. 6.12 Micrograph showing fungal cells present within a degenerating epithelial cell in rat tongue. x 8,500.

Fig. 6.13 *C. albicans* within a lower spinous cell of non-keratinized epithelium. The hypha is surrounded by membranes. x 33,250.

Fig. 6.14 Condensation of tonofilaments around an intracellular hypha in rabbit tongue epithelium (s-collidine buffered osmium). x 30,000.

Fig. 6.15 A connective tissue cell appears to be phagocytosing a fungal cell. x 20,000.

(All tissues except in 6.14 fixed with short term glutaraldehyde & osmium)
unusually dense appearance and their organelles showed degenerative changes such as swollen mitochondria and pyknotic nuclei (fig. 6.12) and occasionally there was a slight disturbance of the cytoplasmic organization in the vicinity of the invading fungi, when condensations of the tonofilaments around the fungal cell wall were seen (fig. 6.14). Frequently, the hyphae present within the deeper epithelial cells appeared to be surrounded by membranes either in single layers or forming more complex structures (fig. 6.13). Hyphae were seen to penetrate from the basal cells through the basal lamina and into the underlying connective tissues. The fungi were often present within connective tissue cells and were surrounded by membranes while some tissue cells even appeared to be phagocytosing the hyphae (fig. 6.15).

6.3.2.3 Fine structure of the host-parasite relationships in vivo

Examination of the specimen from the gingival biopsy material showed _C. albicans_ confined to the superficial epithelial layers as is typical of superficial candidosis. The fungi were present both in and between the superficial cells and no penetration of the deeper cell layers was seen. As intracellular parasites the fungi were present within the cell cytoplasm with tonofilaments abutting directly against the fungal cell wall and no disturbance of epithelial cell organization was seen. The ultrastructural appearance of _C. albicans_ was markedly different from that seen _in vitro_ although it had been fixed in a similar way (according to Rooney and Moens). Organelles and membrane structures were demonstrated with greater clarity (fig. 6.16 & 6.17) and the fungal nucleus with its double membrane was often seen, although the number of mitochondria seemed to be reduced by comparison with the _in vitro_ material. However, the organization of the fungal cell wall was similar to that seen in the _in vitro_ specimens fixed for a short period in glutaraldehyde and osmium; an extensive dense outer layer was not present although extracellular fungi had a flocculent layer of moderate electron density, which became
Fig. 6.16 Candida present in the superficial cell layers of gingival epithelium in vivo. (Short-term glutaraldehyde & osmium). x 3,000.

Fig. 6.17 The clear intracellular detail of a hypha in vivo, a moderately electron dense layer is associated with the outer cell wall. (Short-term glutaraldehyde & osmium). x 31,250.
less apparent when the hyphae were in close association with the epithelial cell cytoplasm. An additional difference was an increase in the amorphous granular material present in the fourth electron lucent layer of the cell wall as compared to the \textit{in vitro} specimens.

6.4 \textbf{DISCUSSION}

Ultrastructural examination of the cultured tissues, both infected and non-infected, confirmed the assumption made on the basis of histological studies that the structural integrity of the epithelium was satisfactorily maintained throughout the experimental period. In the control tissues, at least, the signs of subcellular degeneration that were occasionally seen only seemed to affect a few cells and were often of the type recognized as being reversible (Trump & Arstila, 1975). Such degeneration was probably due to trauma in the preparation of the explants and does not necessarily indicate unsatisfactory conditions of maintenance. Although there was disorganization of the connective tissue and death of some of the cells, the viability of other cells was suggested by their phagocytosis of \textit{C. albicans}. Hill (1978) in ultrastructural studies on rat palate in culture has reported that the connective tissues undergo a "repair" process for the first few days in culture, viable cells phagocytosing dead and dying tissue components.

A fundamental problem in the interpretation of the ultrastructural observations was associated with the determination of the growth phase of \textit{C. albicans} in the tissue sections. As previously mentioned in the results distinction between yeast and mycelial phases on the basis of thickness of the cell wall (Cassone \textit{et al.}, 1973) proved unsatisfactory when \textit{Candida} was present within tissues. The problem in interpretation is a basic one concerning the recognition of structures with different forms from their profiles (see diagram 6.1).
Diagram 6.1 Profiles of sections through a cylinder (hypha) and sphere (yeast).

Most of the fungal profiles were circular or ovoid as would be expected in random tissue sections, only where the hyphae were cut longitudinally could one be sure of the fungal growth phase. However in the histological studies it was seen that the majority of organisms were present in the M-phase and in the ultrastructural studies consecutive thin sections often confirmed that ovoid profiles belonged to hyphal forms of *C. albicans*.

The ultrastructural appearance of *C. albicans* varied considerably according to the methods of fixation used. In the experimentally infected material, the fungal cell wall structure was clearly elucidated particularly when using glutaraldehyde and osmium as fixatives, but details of intracytoplasmic organization were poor. Only in specimens incubated for 40 hours post inoculation and fixed with osmium alone was any clear definition of subcellular structure obtained, and this was very
inconsistent. However in the in vivo material subcellular organization was clearly defined even though the method of fixation (glutaraldehyde followed by osmium) gave no such detail in the in vitro material.

Reports of the fine structure of the yeast form of Candida have suggested that satisfactory elucidation of internal structures generally requires either sectioning of the yeast cells prior to fixation (Borgers and DeNollin, 1974) or removal of the cell wall (Partridge and Drewe, 1974). Borgers and DeNollin (1974) reported that definition of cytoplasmic components is better during the stationary phase than in the growth phase of the organism.

In studies of human oral candidosis by Cawson and Rajasingham (1972) clear demonstration of the internal structure has been achieved using Palade's osmium fixation. The latter workers made various suggestions as to why structure is more readily defined when C. albicans is present within a tissue. One reason they suggested was that the method of fixation may be influential; this is not supported by the present studies where glutaraldehyde and osmium gave better fixation of the human biopsy than of the in vitro tissues. However the best definition of intracytoplasmic detail in vitro was obtained using Palade's fixation and it may be that this particular method is most satisfactory for demonstration of internal structure. Cawson and Rajasingham also suggested that hyphal elongation disperses the organelles and allows better structural definition. This could not be confirmed here for long hyphae present in the in vitro material generally had a dense cytoplasm. Finally it was proposed that glycogen particles present within the fungal cytoplasm might contribute to its dense appearance (Cawson & Rajasingham, 1973) and that invasion of the epithelium may be accompanied by a change in fungal metabolism as a consequence of which there is consumption of the glycogen present, so that the internal organelles are revealed. In the present studies invasion was not accompanied by a loss of dense ground substance although it might be suggested that the time interval over which hyphal invasion was observed was not long enough to allow
significant changes in fungal morphology to have taken place. Such a hypothesis would be supported by the fact that the most satisfactory detail was seen in material cultured for the longest time period.

In the present in vivo material certain structural features suggested that the hyphae present were in the stationary phase of growth (Borgers & DeNollin, 1974). These included the demonstration of nuclei, the appearance of extended invaginations of the plasmalemma and a paucity of mitochondria. In the in vitro material mitochondria were frequently seen, nuclei were rarely seen, if at all, and the plasmalemma showed only short invaginations - all consistent with the growth phase. It thus appears that the variation in ultrastructural appearance of C. albicans in the in vivo and the in vitro material may be accounted for by the difference between fungal organization during the growth and stationary phases.

The appearance of the cell wall of C. albicans, when fixed with osmium is similar in the cultured tissues to that demonstrated in vivo by Cawson and Rajasingham. However primary fixation with gluteraldehyde and post-osmication, especially when prolonged, produced better definition of the cell wall, especially of the outer floccular layer and the structure is similar to that demonstrated by Djazenko and Cassone (1972) in the yeast form of C. albicans. Cassone et al. (1973) have reported changes in the morphology of the cell wall during budding and in the present in vitro tissues the cessation of the fibrous third layer and expansion of the fourth layer in the budding wall was in accord with their observations. However changes in the outermost layers were less obvious; such changes may have been missed since a sequential study of budding within tissues could not be made, however the intra-epithelial environment may greatly influence the behaviour of fungi and induce further changes in the cell wall structure. In the present studies changes in the outer layers of cell wall of the invading fungi appeared to be associated with the invasive process.

Montes and Wilborn (1968) were the first to comment on the thick layer of extracellular material adherent to the external surface of the
cell wall of *C. albicans* which appeared to become detached as the fungus invaded the epithelial cell, and reports of a similar coating have been made in fungi infecting chick chorio-allantoic membrane (Cawson & Rajasingham, 1973) in studies of phagocytosis of *C. albicans* by polymorphs (Belcher et al., 1973) and in Candida infected urine samples (Müller et al., 1977). In the present studies of in vivo infected tissues a similar material was observed around extracellular fungi although it does not appear to correspond to the dense outer floccular cell wall layer of *C. albicans* seen in vitro, being generally more extensive, amorphous and less dense in appearance. Müller et al. (1977) suggest this coating corresponds to the "asteroid body" which has been described for *C. albicans* and other fungi (Lurie, 1963; Berge & Kaplan, 1966) and which is seen histologically as eosinophilic material radiating from around the fungal cells. Several theories have been advanced as to the origin of the substance; - that it is either host derived, or is a protective product of the fungus, or even develops from both the host and parasite (Berge & Kaplan, 1966). Lurie (1963) suggested that the material is composed of antigen-antibody complexes precipitated on the cell wall of fungi in the parasitic condition, and this has been supported by immunochemical studies (Berge & Kaplan, 1966; Müller et al., 1977). While it could be suggested that the amount of this material may be too extensive to consist entirely of such complexes, it seems possible that salivary or serum factors from the systemic environment may contribute to the material. This would explain why a similar layer is not present in the in vitro material.

The ultrastructural relationship established between *C. albicans* and oral mucosa demonstrated in vitro was similar in all the specimens, the studies provided a considerable amount of information about the host-parasite relationship. In the superficial epithelial layers the relationship was similar to the previous reports of in vivo infections (Montes & Wilborn, 1968; Cawson & Rajasingham, 1972; Mohammed, 1975). However some conflict exists in the literature regarding the extent of host cell disorganization by the invading fungi. Cawson and Rajasingham
(1972) reported a complete lack of host cell disorganization by the intracellular hyphae, while Montes and Wilborn (1968) and Mohammed (1975) demonstrated a loss of tonofilaments adjacent to some intracellular fungi. The latter workers suggested that this might represent keratolytic activity on the part of C. albicans. In the present studies slight changes in cytoplasmic organization were sometimes seen in close proximity to the fungal cell wall. This was however seen in material in which fixation failed to demonstrate the thick outer cell layer and it might be suggested that the unrecognized presence of this structure may affect the appearance of the cytoplasm adjacent to the fungal cell wall. More striking however, was the occasional but complete loss of cell cytoplasm around invading hyphae. A number of explanations may account for these spaces. They may represent shrinkage artefact, although as it often only affected one hypha in a group this would seem unlikely. However it is suggested that in fungal hyphae the tips of the hyphae are plastic and the rest of the cell walls are rigid (Burnett, 1968) thus differential shrinkage of the structure may occur. On the other hand the spaces could be the site of enzyme digestion of the epithelial cell cytoplasm. It has been suggested that, in general, secretion of enzymes from hyphae is only likely to occur at the apical tip (Chang & Trevitick, 1974). The more 'acute' nature of the invasive process in the present experiments may account for the more complete loss of cell cytoplasm compared with that demonstrated by Montes and Wilborn (1968) and Mohammed (1975).

Although it is convenient to postulate that C. albicans secretes enzymes capable of dissolving the epithelial cell cytoplasm there was little morphological evidence that such a mechanism played a part in the penetration of the epithelial cell membrane. No gross disturbance of the cell membrane was seen adjacent to invading hyphae, except in one case where the plasma membrane of a keratin squame was seen to be discontinuous at the site of fungal entry. Montes and Wilborn reported this as a regular occurrence, however it is felt that this is a reflection of the effete nature of surface squames rather than a feature of fungal invasion. In the present experiments both transmission and scanning
micrographs confirmed that a very close seal existed between the plasma membrane and fungal cell wall. Enzymatic breakdown of the cell membrane, if occurring, must be closely localized to areas of immediate contact between the epithelial membrane and fungal wall. The considerable amount of distortion of epithelial cell membranes seen most often in fungal invasion of non-keratinized mucosa would suggest that a considerable amount of pressure is exerted by the fungi in their growth through cells and that this may contribute to the invasive mechanism.

While the presence of Candida in the superficial epithelial cells appeared to elicit no response in these cells, the occurrence of membrane bound hyphae in the deeper epithelial cells suggests these cell are more responsive to fungal invasion. It must be pointed out that this appearance may be due to sectioning through a distorted membrane associated with an invading hyphae although this seems unlikely as it was a relatively frequent occurrence. Belcher et al. (1973) demonstrated the phagocytosis of C. albicans by polymorphonuclear leucocytes at the ultrastructural level. In the present studies connective tissue cells in the tissues in vitro were seen to surround C. albicans with phagocytic membranes and it seems possible that epithelial cells are also capable of producing membranes around the fungus in an effort to exclude it from the cell. Phagocytosis of foreign material by epithelial cells in vivo has been described (Wolff & Schreiner, 1970). This will be discussed further in Chapter 9.

The association of C. albicans with cells of the nucleated epithelium which appeared to be degenerating suggests that the fungi may have had a more damaging effect as intracellular parasites on the deeper epithelial cells than was observed in the superficial cells. However the control, non-infected tissues also contained a few cells showing degenerative changes, so whether C. albicans is capable of rapidly killing nucleated epithelial cells or merely has a predilection for cells undergoing incipient degeneration remains a matter for consideration. That Candida may invade certain cells is supported by the apparent predilection for glycogen rich superficial cells seen in the infected rabbit cheek mucosa.
6.5 CONCLUSIONS

From these studies it appears that at the ultrastructural level invasion of the oral epithelium in vitro is similar to that seen in vivo and that the superficial epithelial cells appear unaffected in the short term by the presence of C. albicans. In the cultured tissues C. albicans enters the deeper epithelial cells and evidence suggests that these cells may respond to the presence of fungi by the production of phagocytic vesicles around the intracellular hyphae. Disruption of the epithelial cell membranes by invading fungi was minimal although it appeared that both enzymatic and mechanical forces may be involved in epithelial invasion by C. albicans. Changes in the ultrastructural organization of the fungal cell wall also appeared to be related to the invasive process.
CHAPTER 7

CYTOCHEMICAL STUDIES ON THE HOST-PARASITE RELATIONSHIP IN VITRO

7.1 INTRODUCTION
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  7.3.2 Localization of phospholipase
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7.1 INTRODUCTION

A number of hydrolytic enzymes have been found in association with *C. albicans* grown in culture but there are no reports of the enzyme activity of the fungi within tissues. Hydrolytic enzymes, by breaking down cell membranes, and possibly intercellular material could facilitate the penetration of epithelial tissues by *C. albicans* and two such enzymes which might play a role in invasion are acid phosphatase and phospholipase.

Acid phosphatases consist of a group of enzymes which hydrolyze a variety of organic esters at acid pH. Chattaway et al. (1971) demonstrated the presence of a number of acid phosphatases in *C. albicans* using biochemical techniques; these enzymes had pH optima between 3.6 and 5.6, and, at pH 4.0, the activity was associated specifically with the cell wall fraction of the fungi. Marriott (1976), studying isolated plasma membranes, found little phosphatase activity associated with these membranes at pH 3.6. DeNollin et al. (1975) have demonstrated the presence of acid phosphatase at the ultrastructural level in the vacuoles of *C. albicans* although no activity was seen in the region of the cell wall.

The phospholipases are also hydrolytic enzymes which have as their substrate the phospholipids commonly present in plant and animal membranes. Clearly such enzymes could play an important role in facilitating the entry of fungus into cells and Pugh and Cawson (1975) were the first to suggest that such enzymes may be active in the invasion of epithelial cells by *C. albicans*. Although Chattaway et al. (1971) had failed to demonstrate the presence of phospholipase biochemically in either the yeast or mycelial forms of the fungus, Costa et al. (1967-1968, cited in Pugh & Cawson, 1975) reported the presence of phospholipases A and C in *C. albicans* (four types of phospholipases, A-D, are recognized according to the site of action of the enzyme on the substrate molecule). More recently Pugh and Cawson have developed a method for the cytochemical demonstration of phospholipase A and described its ultrastructural location in the fungi (1975).
The following experiments used the cytochemical methods applied by DeNollin et al. (1975) and Pugh and Cawson (1975) to investigate the activity of acid phosphatase, phospholipase in C. albicans penetrating epithelial tissues in culture.

7.2 MATERIALS AND METHODS

Tissues for the cytochemical studies consisted of explants of neonatal rabbit tongue infected with C. albicans strain 3153 and non-infected control explants. These were prepared and maintained under the conditions described in Chapter 4. The specimens were harvested 12 hours after inoculation and fixed and incubated according to the following methods.

7.2.1 Demonstration of acid phosphatase activity

Tissues were fixed in half strength Karnovsky fixative buffered with 0.1 M cacodylate (Karnovsky, 1965) at pH 7.2 and containing 2 mM calcium chloride to stabilize membranes, for 3 hours at 4°C. They were then washed overnight in buffer containing calcium chloride. After agar embedding, the tissues were chopped into sections of approximately 50μ thickness using a Sorvall tissue chopper. The tissue pieces were incubated in freshly prepared media containing either sodium beta-glycerophosphate (Barka & Anderson, 1963) or para-nitrophenylphosphate (DeNollin et al., 1975) as substrate and lead nitrate as the capture agent at pH 5.0. Control tissues were carried through at the same time in complete incubating media to which 10 mM stannous fluoride was added. After 1 hours incubation at 37°C, the tissues were washed in buffer, and fixed in cacodylate buffered osmium tetroxide for 1½ hours, rinsed, dehydrated and embedded in Epon. Ultrathin sections were cut and examined in the electron microscope.

7.2.2 Demonstration of phospholipase A activity

The specimens were first washed in saline then fixed in 2.5% cacodylate buffered formaldehyde at pH 7.2 containing 2 mM calcium
chloride for 40 minutes at 0-4°C. After washing in buffer solution containing calcium chloride for 1 hour the tissues were embedded in agar, chopped into 50μ slices and briefly washed in 0.05 M acetate buffer. They were then incubated at 37°C. for 45 minutes in one of the following freshly prepared media:

i) A medium based upon that of Pugh and Cawson (1975) and containing 9 parts 0.2% lead nitrate in 0.05 M acetate buffer pH 5.0 and 1 part of 1.0 mg/ml phosphatidylcholine in 0.1% Triton-X-100.

ii) A control medium to verify the method of demonstrating phospholipase activity, using sections of non-infected tissues. This consisted of the same medium as above plus 0.02 ml of phospholipase A (Sigma Chemical Co. U.S.A.) per ml of medium. The rationale for this procedure was that the phospholipase present in the medium would liberate fatty acids from the phospholipids in the tissues, which would be localized by the lead.

iii) A control medium to evaluate the affinity of lead nitrate for the tissues consisting only of 0.2% lead nitrate in acetate buffer at pH 5.0.

The constituents of the incubation media containing phosphatidylcholine (i & ii) were immiscible. This would be likely to cause uneven penetration of the cytochemical agents and could lead to artefactual precipitation of lead in the tissues (Essner, 1973). Thus the media were lightly sonicated for about 5 minutes to produce a fine dispersion. Although reports in the literature indicate that phosphatidylcholine is degraded by prolonged sonication, that carried out was of short enough duration to avoid this (Hauser, 1971).

After incubation the tissue pieces were washed in buffer, postfixed in osmium and prepared for electron microscopy as above.
Fig. 7.1 A micrograph of an infected explant incubated to demonstrate the presence of acid phosphatase. Reaction product is present within the lysosomes in an epithelial cell (beta-glycerophosphate as substrate). x 7,350.

Fig. 7.2 In the demonstration of acid phosphatase, non-specific reaction product occurred over some epithelial cell nuclei (N) and the cytoplasm of certain superficial epithelial cells (C), while product was also located intercellularly in the upper granular and lower keratin cell layers (para-nitrophenylphosphate as substrate). x 6,600.
7.3 RESULTS

7.3.1 Localization of acid phosphatase

No great difference was seen after incubation of the tissues in the two different substrates, beta-glycerophosphate and para-nitrophenyl-phosphate although the latter appeared to give more non-specific background staining of the epithelium. In the control tissue, incubated with sodium fluoride there was complete inhibition of the reaction.

Sections of non-infected epithelium showed lead phosphate reaction product localized in a number of sites in the keratinized tissue. In all cell layers except for the superficial keratin layer reaction product was observed in membrane bounded vesicles 0.3 μm - 0.5 μm in diameter which resembled lysosomes as described in a number of reports (Waterhouse & Squier, 1966; Silverman & Kearns, 1970; Squier, 1971) (fig. 7.1). In the cells of the granular layers small membrane bound vesicles containing reaction product were present adjacent to the superficial plasma cell membranes, these corresponded in size and location to membrane coating granules (Weinstock & Wilgram, 1970; Gonzales et al., 1976). A considerable quantity of reaction product was also seen intercellularly between the cells in the upper granular and lower keratin layers, where it was clearly demarkated by the plasma membranes of the adjacent cells (fig. 7.2). Certain of the superficial cells in the keratin layer showed extensive granular reaction product in the cytoplasm, while a lead precipitate was also seen over some of the nuclei of the epithelial cells (fig. 7.2).

The epithelium of infected explants showed a similar distribution of reaction product. Fixation of C. albicans was poor; both intra and extracellular fungi showed little evidence of reaction product, many hyphae showing no lead precipitate at all. When present, the reaction product was mainly confined to central vacuoles and the membranes although occasionally smaller apparently membrane bounded intracytoplasmic vesicles were evident (fig. 7.3). In most cases there was little evidence of cell wall staining in the fungi in the superficial epithelial cell
Fig. 7.3  Reaction product localized to membranes of intracytoplasmic vacuoles of *C. albicans* in an explant incubated to demonstrate acid phosphatase activity (beta glycerophosphate as substrate). x 20,400.

Fig. 7.4  Extracellular hyphae of *C. albicans* showing small granules of reaction product within the central vacuole of the hypha and associated with the outer cell wall (beta glycerophosphate as substrate). x 34,000.
Fig. 7.5  In the explants incubated to demonstrate the presence of phospholipase A, reaction product was present intercellularly between cells of the upper granular and lower keratin cell layers. x 4,500.

Fig. 7.6  In the explants incubated with substrate and phospholipase localization reaction product was unchanged. x 4,500.
layers, but where breakdown of the epithelial cells and their membranes was evident small granules of reaction product could be seen associated with the outer cell wall of the invading hyphae (fig. 7.4). In the control tissues prepared by adding sodium fluoride to the incubating medium almost complete inhibition of enzyme activity in the fungi occurred, although some non-specific reaction in the fungal cell wall was seen.

7.3.2 Localization of phospholipase

The localization of lead precipitates within the epithelium was similar in both experimental and in control tissues incubated without substrate. Occasionally some staining of the cell nuclei was evident but otherwise very little background staining was seen in either the experimentals or control. The lead product was seen as granular deposit bounded by the adjacent plasma membranes between the cells of the granular and deeper keratin layers (fig. 7.5). Tissues incubated in medium with phospholipase added showed no more activity than the experimental tissues, the most conspicuous reaction occurring between the cells of the granular and deeper keratin layers (fig. 7.6).

In the infected tissues incubated to demonstrate phospholipase activity, reaction product was associated with some but not all of the fungi, and in these was present in a number of locations. Product was seen in small, discrete, vesicles localized either in groups or distributed throughout the cytoplasm (fig. 7.7) or localized to membranes of larger vesicles and other membranous organelles; the quality of fixation was not sufficient to allow identification of such organelles. No extensive localization of enzyme was found over the fungal cell wall but there were some well localized deposits (fig. 7.8). Small granules and vesicular deposits also appeared to be associated with the outer cell wall in hyphae which are either intracellular or in close association with the epithelial cells (fig. 7.9). A similar location of reaction product has been demonstrated in *C. albicans in vitro* (Pugh & Cawson, 1975).
In the infected explants incubated to demonstrate phospholipase activity, reaction product was present in a number of locations within intracellular fungi: in small intra-cytoplasmic granules, Fig. 7.7 (22,500), in localized areas of the cell wall, Fig. 7.8 (x 19,500) and in the outer cell wall region, Fig. 7.9 (x 38,000).
Lead precipitate was not present around the periphery of all intracellular hyphae, nor were heavy deposits of reaction product regularly seen to be present at the site of penetration of the epithelial cell membrane. However as in fig. 7.9, it can be seen that enzyme activity was sometimes associated with membrane penetration.

In the control tissues incubated without substrate some reaction product was also present in similar locations to the experimental tissues (fig. 7.10).

Fig. 7.10 Reaction product present both within the fungal cell and associated with the outer cell wall in an explant incubated without substrate as a control for the demonstration of phospholipase. $\times$ 20,700.
7.4 COMMENTS ON CYTOCHEMICAL METHODS

The ultrastructural demonstration of enzymes by metal precipitation methods is accompanied by various artefacts which affect the resolution and complicate the interpretation of sites of enzyme activity. Satisfactory results depend upon the fine structure of the tissue being adequately preserved, despite incubation of the material in chemically and osmotically destructive media. Fixation may ensure such preservation and limits enzyme diffusion but it also inhibits enzyme activity. Such difficulties must be borne in mind when interpreting the results of the previous experiments and in considering the findings of other workers in the field.

The cytochemical demonstration of acid phosphatase activity was based upon Barka and Anderson's method (1963) and uses lead to trap the phosphatase ions liberated by the enzymatic hydrolysis of organic ester substrates. The resulting precipitate is electron dense and can be seen at the ultrastructural level. Two substrates were used in these studies, for DeNollin et al. (1975) found nitrophenylphosphate was a more satisfactory substrate than glycerophosphate. However little difference was seen between the two substrates in the present experiments. Among the artefacts often associated with this well established technique is that of scattered deposits of non-specific reaction product throughout the tissue (Essner, 1973). Goldfischer et al. (1964) suggested that such random precipitates are probably derived from the hydrolytic action of soluble (unfixed) enzyme or by diffusion of lead phosphate away from the sites of enzyme activity. Essner (1973) also reported that the free lead present in the incubation media can be preferentially absorbed upon organelles, simulating enzyme localization. Barka and Anderson (1963) suggest that with an excess of sodium beta-glycerophosphate the lead salt may be formed which has an affinity for the nucleus, and this may account for some of the nuclear staining seen in the present studies.

In the demonstration of phospholipase A the substrate phosphatidylcholine is hydrolysed by phospholipase A into lysophosphatidylcholine and
a fatty acid. It is claimed that another enzyme, lysophospholipase, then further splits the lysophosphatidylcholine into glycerophosphorylcholine and a fatty acid (Pugh & Cawson, 1975). In the method of Pugh and Cawson enzyme activity is localized by coupling the released fatty acids with lead. The reaction product formed when phosphatidylcholine is used as substrate will therefore reflect the activity of both enzymes. It is believed that lysophosphatidylcholine causes much damage to membranes, and the breakdown of this compound by the lysophospholipase limits the amount of membrane degradation taking place (Pugh & Cawson, 1975).

The detergent Triton-x-100, a non-ionic detergent, added to the incubation media encouraged dispersion of substrate. It has been reported to stimulate phospholipase activity, although only when present at higher concentrations than those used in the present investigations (Scandella & Kernberg, 1971). Its influence on the reaction may also be questioned since it has been shown that detergents may release the endogenous membrane bound phospholipase, which has been identified in a number of animal and microbial membranes (Scandella & Kernberg, 1971; Victoria et al., 1971); Scandella and Kernberg (1971) defined the level at which Triton-x-100 released significant amounts of the bound phospholipase as 0.5 mg/ml whereas in the present studies only 0.1 mg/ml was present. Provided there was even penetration of tissues this level might not be expected to labilize membrane bound enzymes.

7.5 DISCUSSION

The localization of acid phosphatase activity in lysosomes, membrane coating granules and the adjoining intercellular spaces seen in these studies agrees with previous work on enzyme localization in oral mucosa (Squier & Waterhouse, 1970; Weinstock & Wilgram, 1970) as does the diffuse staining of apparently random superficial epithelial cells (Susi, 1968; Silverman & Kearns, 1971). The localization of phosphatase within the cytoplasmic vacuoles of C.albicans was generally in agreement with the findings of DeNollin et al. (1975) although in the present studies
enzyme was found more closely associated with the membranes of the vacuoles. It is possible that enzyme activity may differ between yeast cells in vitro, studied by DeNollin et al. (1975) and hyphae within tissues. There was no evidence in the present studies of cell wall localization except for that associated with the extracellular 'coat'.

The demonstration of an acid phosphatase associated with the cell wall of C. albicans by Chattaway et al. (1971) supports this observation while the weakness of the cytochemical reaction might be attributed to the difference between the optimum pH at this site claimed by Chattaway et al. (pH 3.6-4.0) and the pH of 5.0 at which the cytochemical studies were carried out. Furthermore aldehyde fixation has been shown to inhibit the enzyme (Essner, 1973; Bauer & Sigarlakie, 1973). Alternatively it could be suggested that the reaction product was not associated with the fungi but with the epithelial cells which were in contact with the fungi.

In examining the distribution of phospholipase activity the localization of enzyme intercellularly, between the granular and deeper keratin layers, was not altogether an unexpected finding. This is the region of the epithelium where the membrane coating granules have been claimed to discharge their contents into the intercellular spaces (Lavker, 1976) and phospholipids and lipids have been identified among their constituents (Susuki & Kurosumi, 1972). It is reasonable to assume that the modification of these substances by hydrolytic enzymes, also contained within the granules (Gonzalez et al., 1976) may release free fatty acids and lipids which react with the lead.

The association of phospholipase activity with the fungi present within the epithelium was in many instances similar to the localization demonstrated in yeasts in vitro by Pugh and Cawson (1975) although rarely was the reaction product as intense. These workers attributed the varying distribution of the enzyme activity to stages in the budding and development of the yeast. When C. albicans is present within epithelium, as in the present study, such sequential development cannot be followed although among individual fungi the same range of distribution of enzyme activity can be seen. The absence of heavy deposits around extracellular yeast
cells, taken by these workers to represent autolysis of the fungi, might suggest that many of the fungi remain viable in the \textit{in vitro} system. The localization of reaction product in small granules in the cell 'coat' of the hyphae was similar to that found by Pugh and Cawson (1975) in yeast cells \textit{in vitro}. Although in the present studies such granules were present around intracellular hyphae there is no firm evidence to associate phospholipase activity with penetration of the epithelial cell membranes.

The major problem in interpreting these results was the similar staining seen in experimental and control tissues and a number of factors could be suggested to account for this. Firstly, the control specimens were incubated without substrate and no specific enzyme inhibition was used. Thus it is possible that endogenous substrate might be available and would account for staining in these controls. Pugh and Cawson (1975) commented on the staining obtained in cells incubated without substrate which they attributed to the hydrolysis of lipids present in the cells. They reported that such non-specific staining was reduced by adequate washing. In the present studies the tissues were washed for considerably longer periods than those used by Pugh and Cawson, so this would not appear to be a satisfactory explanation. On the other hand localized staining of the fungal plasma membrane and adjacent cell wall was seen in some of the invading hyphae in the control preparations. Marriott (1976) has demonstrated the presence of free fatty acids in the plasma membrane of \textit{C. albicans}, and these may lead to such non-specific staining.

More recently Pugh and Cawson (1977) have published a study on the phospholipase \textit{A} activity in \textit{C. albicans} infecting the chick chorio-allantoic membrane and described heavy enzyme activity associated with the invading hyphae, especially at the site of membrane penetration. Once again the intensity of reaction product was considerably greater than in the present studies. While it is reasonable to suggest that uniform penetration by the incubation media may be less readily achieved in oral epithelium than in the respiratory epithelium of the chick membrane it is difficult to find other valid explanation for this discrepancy.
7.6 CONCLUSIONS

The cytochemical localization of two hydrolytic enzymes within the infected explant showed that the presence of acid phosphatase in epithelium is similar to that seen in vivo and that phospholipase A may be present intercellularly in the upper spinous and lower keratin layers of neonatal rabbit tongue epithelium.

In the invading fungi phosphatase was predominantly localized within intracytoplasmic vacuoles, however some enzyme activity was associated with the outer cell wall of the hyphae. This may represent secreted fungal enzymes or may originate from the host epithelial cells. Phospholipase was demonstrated in a number of locations both intracellularly and in association with fungal cell wall. It is suggested that these enzymes may play a role in the breakdown of the epithelial cell cytoplasm but their role in penetration of the epithelial cell membranes cannot be definitely established.
CHAPTER 8

IMMUNOLOGICAL STUDIES ON THE IN VITRO MODEL OF CANDIDAL INFECTION

8.1 INTRODUCTION

8.2 MATERIALS AND METHODS

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8.2.1.1 Preparation of antigen
8.2.1.2 Immunization of rabbits
8.2.1.3 Extraction of IgG
8.2.1.4 Double diffusion test
8.2.1.5 Immunoelectrophoresis

8.2.2 Incorporation of immune, non-immune sera and IgG in the model system

8.2.2.1 Preparation of the media
8.2.2.2 The in vitro system
8.2.3.1 Test for the presence of anti-candidal antibodies in serum
8.2.3.2 Indirect test for the presence of Candida antigens in infected explants
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8.3.5.3 Direct test for the presence of anti-candidal antibodies in infected explants

8.4 **DISCUSSION**

8.5 **CONCLUSIONS**
8.1 INTRODUCTION

The invasion of oral mucosa in vitro by C. albicans differs from invasion in vivo in one major respect, the depth of penetration of the epithelium by the fungus. The studies related in Chapters 4 and 6 suggest that the unrestricted invasion of C. albicans in vitro does not result from changes in either the epithelium as a whole or in individual epithelial cells. It therefore seems possible that factors absent in vitro, and probably of systemic origin influence the pattern of invasion in vivo. The following studies set out to examine the effect of such systemic factors as immune and non-immune serum, and immunoglobulins on the in vitro model.

8.2 MATERIALS AND METHODS

8.2.1 Preparation of Immune and Non-immune sera

8.2.1.1 Preparation of antigen

C. albicans strain 3153 was used to prepare the following antigenic suspensions in normal saline:

a) \(5 \times 10^5\) killed Y-forms per ml.

b) \(3.5 \times 10^6\) killed Y-forms and M-forms per ml.

c) A very thick suspension of sonically disrupted Y and M forms.  
   (Full details of the preparation are given in Appendix 2)

Antigen solutions were stored at -20°C.

8.2.1.2 Immunization of rabbits

One white New Zealand, male rabbit was immunized via an intra-venous route according to the following schedules:

Antigen (a); 1 ml at 1, 4, and 7 days and 2 ml at 14 days.

Antigen (b); 1 ml at 4, 5, and 6 weeks and 2 ml at 7 weeks.

Antigen (c); 0.5 ml at 11, 13, and 15 weeks.

The rabbit was test bled (10 ml of blood from ear vein) before immunization and again at 9 weeks and 16 weeks.
The immunized rabbit together with a control unimmunized rabbit were finally bled by cardiac puncture at 17 weeks. The blood was collected in a sterile bottle and left to clot at +4°C. overnight. The sera were then collected and centrifuged at 12,000g for 30 minutes to remove large particles of fibrin, cell debris and lipoprotein and stored at -20°C.

8.2.1.3 Extraction of IgG

The IgG fraction of the immunoglobulin was extracted from some of the immune sera by ion exchange chromatography on Sephadex gel prepared by the method of Joustra et al. (1969) using 0.1M ethylene diamine/acetic acid buffer at pH 7.0. Full details of this preparation are given in Appendix 2.

About 10 mls of immune serum was dialysed against ethylene diamine/acetic acid buffer for 3 days at +4°C. The dialysed serum was poured onto the column, and after discarding the bed volume the protein fractions were collected and stored at +4°C. A 0.1M acetate buffer pH 4.0 was then run through the column in the cold to extract the remaining protein fractions.

8.2.1.4 Double diffusion test

Serum from each of the test bleeds was examined for antibodies to C.albicans by gel precipitation tests using the Ouchterlony double diffusion method. Plates were poured with Ionagar in barbitone acetate buffer pH 8.6 and the well template follows that of Odds et al. (1975) (see figure 8.1).

The filtrates of the original antigen suspensions were used as antigen. Diffusion was carried out in moist chambers at room temperature for 3 days. The plates were then stained with Coomassie brilliant blue R.

8.2.1.5 Immunoelectrophoresis

The immunoelectrophoresis was run using 1% Agarose gel (B.D.H. Chemicals Ltd., England) in 0.02M barbital buffer pH 8.6. The process was carried out in an L.K.B. 2117 Multiphase electrophoretic model. The
Table 8.1

<table>
<thead>
<tr>
<th>Medium</th>
<th>Nos. of Explants</th>
<th>Incubation period in hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Infected</td>
</tr>
<tr>
<td>Dorsal mucosa of rabbit tongue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T.C.M.</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>T.C.M. &amp; 20% non-immune serum</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>4(1)</td>
<td>7(2)</td>
</tr>
<tr>
<td>T.C.M. &amp; 20% immune serum</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>4(1)</td>
<td>7(2)</td>
</tr>
<tr>
<td>T.C.M. &amp; 20% lgG</td>
<td>2(1)</td>
<td>5(1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5(2)</td>
</tr>
</tbody>
</table>

(figures in brackets represent explants processed for immunofluorescence).
well template is shown in fig. 8.2 and the wells were filled with:

a) Original immune serum.
b) IgG fraction of immune serum.
c) IgG diluted with tissue culture media.
d) Remaining serum protein fraction of immune serum.

Using these wells as the anode a current of 30 volts was passed for 30 minutes at a gradient of 7-8 volts per cm.

Sheep anti-rabbit immunoglobulin (Wellcome Reagents, Kent) was placed in the troughs (see fig. 8.2) and the plate stored at 4°C. for 72 hours. After washing the plate was dried and stained with Coomassie brilliant blue R.

8.2.2 Incorporation of Immune, Non-immune sera and IgG into the Model System

8.2.2.1 Preparation of the media

The extracted IgG was dialysed against Tissue Culture Media for 3 days at 4°C. and both the dialysed IgG and the sera were sterilized by filtration through a 0.22 μm pore Millipore filter.

The nutrient media were prepared as follows:

a) Normal tissue culture media (T.C.M.) as in Chapter 4.
b) T.C.M. with 20% immune serum.
c) T.C.M. with 20% non-immune serum.
d) T.C.M. with 20% dialysed IgG.

(All media contained standard doses of penicillin and streptomycin).

8.2.2.2 The In Vitro System

Explants of dorsal mucosa of neonatal rabbit tongue were prepared and maintained as in Chapter 4, employing nutrient media according to the regime set out in table 8.1.

All explants, except for controls, were inoculated after 4 hours maintenance with C. albicans 3153 by means of wire as in Chapter 4.
Tissues were harvested after 8 or 12 hours and then fixed and prepared for light microscopy as described previously or quick frozen in isopentane and liquid nitrogen for immunofluorescent studies.

8.2.3 Immunofluorescent Studies on the Infected Tissues

8.2.3.1 Test for the presence of anticandidal antibodies in serum

Smears of *C. albicans* 3153 grown overnight in T.C.M. were made on teflon-coated slides. These were then reacted with a series of dilutions (1:5, 1:10, 1:20) of either the non-immune serum, the immune serum or the IgG fraction. After washing the smears were reacted with fluorescein conjugated sheep anti-rabbit globulin in a moist atmosphere. (Details of this and the following tests are given in Appendix 2).

8.2.3.2 Indirect test for the presence of Candida antigens in infected explants

5 μ sections of explants of rabbit mucosa maintained in T.C.M. and infected with *C. albicans* were cut at -15°C., in a cryostat, collected on a coverslip and left to dry at room temperature. The 'indirect' or 'sandwich' technique was then employed for the immunofluorescent demonstration of *Candida* antigens. The tissue sections were washed and reacted with a 1:10 dilution of the rabbit sera or IgG fraction, rewashed and then reacted with the fluorescein conjugated antibody. Unwashed control specimens were treated either with the IgG fraction followed by the fluorescent antibody or with the latter alone.

8.2.3.3 Direct test for the presence of anticandidal antibodies in infected explants maintained in T.C.M. with antisera

Frozen sections of infected rabbit mucosa which had been maintained in T.C.M. containing either immune, non-immune sera or IgG were prepared as in section 8.2.3.2. The direct immunofluorescent technique was used to demonstrate the complexing of anti-candidal antibodies with the candidal antigens in the tissues. Washed sections were reacted with fluorescent anti-rabbit immunoglobulin.
All sections from the preparations were mounted in glycerine and water and stored at +4°C. After 2 hours they were examined in a Zeiss Photomicroscope I equipped with an HBO 200 mercury vapour source and an ultraviolet barrier filter orange 500 and absorption filter B.G.12.

8.3 RESULTS

8.3.1 Immunodiffusion Test

Fig. 8.1 is a photograph of the Ouchterclony plate used to test for the presence of antibodies to C. albicans in the serum from both immunized and non-immunized rabbits. Lines of precipitation can be seen between the immune serum and antigen only. Non-immune serum did not react.

8.3.2 Immunoelectrophoresis

Fig. 8.2 is a photograph of the immunoelectrophoresis plate. The presence of rabbit immunoglobulins was confirmed in all the fractions tested including the extracted IgG fraction, and it was demonstrated that this activity was still present when the fraction was diluted with culture media.

8.3.3 Estimation of the Titres of Complement Fixing Antibodies in the Culture System

In order to have an estimate of the antibody titres present in the in vitro system, complement fixation tests were carried out on the nutrient media by the Clinical Laboratory Service of the London Hospital. The titres of the complement fixing antibodies to C. albicans present in the culture media with 20% immune serum or IgG was reported to be 1 in 160.

8.3.4 The Effect of Sera and IgG on the In Vitro Model

8.3.4.1 Control Tissues

Maintenance of the uninfected tongue mucosa was satisfactory, irrespective of the type of media used to maintain the explants.
**Fig. 8.1** Photograph of the stained immunodiffusion plate showing the formation of antigen-antibody complexes between the immune serum fractions and the antigen. (ag=antigen, s=immune serum, ns=non-immune serum, lg=extracted lgG).

**Fig. 8.2** Photograph of the stained immunoelectrophoresis plate. A=immune serum, B=lgG fraction, C=20% lgG fraction in T.C.M., D & E other protein fractions extracted from the immune serum. T=fluorescein conjugated sheep anti-rabbit immunoglobulin.
8.3.4.2 Infected tissues

The infected explants maintained by culture media alone showed a similar pattern of candidal invasion as had been seen previously (Chapter 4). The addition of serum, whether immune or non-immune, or of IgG to the media did not appear to influence the activity of *C. albicans*. No difference was observed in the proportion of fungal Y-forms to fungal M-forms in or on the tissues, or in the pattern of candidal invasion.

8.3.5 Immunofluorescent Studies

8.3.5.1 Indirect test for candidal antibodies in serum

It can be seen from table 8.2 that all smears of *C. albicans* treated with immune serum or IgG showed some degree of immunofluorescence; significant titres of anti-candidal antibody were present in the immune serum and extracted IgG. Positive fluorescence was also obtained with the undiluted and 1 in 5 dilution of the non-immune serum. This was not taken as a false positive for it is likely that rabbits have immunoglobulins to 'Yeast' antigens. Various species of yeast-like fungi are present in the natural environment of the rabbit and may give rise to non-specific cross-reacting antibodies. As the cross-over point from positive to negative for the non-immune serum occurred at 1 in 10 dilution, while the fluorescent of the immune, and IgG treated *C. albicans* was still clearly positive at this concentration, this dilution was chosen for use in the immunofluorescent studies on infected tissues.

Table 8.2 Showing the relative strength of immunofluorescence of *C. albicans* stained with immune, non-immune sera and IgG

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Immune Serum</th>
<th>Non-immune Serum</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>1:5</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>1:10</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1:20</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
8.3.5.2 Indirect test for the presence of *C. albicans* in infected explants

Those sections stained with fluorescent anti-rabbit immunoglobulin served as controls for estimation of the amount of non-specific background stain. Despite the fluorescent anti-immunoglobulin being directed against rabbit globulin, and the tissues used in the experiments being rabbit mucosa, the non-specific staining was within acceptable limits. Washing sections in buffered saline is generally considered to remove some non-specific staining, but little difference could be seen in the staining of the washed and unwashed specimens.

The presence of *C. albicans* within the tissues was ascertained by dark field illumination, and all specimens were found to be positive for the fungus. On examination in ultraviolet light no fluorescence of *Candida* was seen in those specimens treated only with fluorescent anti-rabbit globulin. This demonstrates firstly, that there was no cross reactivity between the antiglobulin and *Candida* antigens, and, secondly, that anti-candidal rabbit immunoglobulins were not normally present in the tissues in vitro. In those specimens treated with immune or non-immune serum or IgG *C. albicans* was seen to be strongly fluorescent thus demonstrating the presence of anti-fungal antibodies conjugated with the *Candida*. Differences in the strength of the fluorescence between the immune, non-immune sera and IgG were assessed subjectively and one major difference in the pattern of fluorescence between the specific immune and non-immune fractions emerged. When sections were stained with non-immune serum fluorescence of the fungal cell wall was clearly demonstrated, little staining intracellularly or outside the fungi could be seen. However when specific anti-candidal immunoglobulins were present staining of diffuse material outside hyphae was apparent, besides heavy staining of the fungal cell wall (figs. 8.3, 8.4).

8.3.5.3 Direct test for the presence of anti-candidal antibodies in infected explants

On examination of specimens with ultraviolet light non-specific background staining of the tissues was seen to be within acceptable limits.
Fig. 8.5 A section from an explant cultured in media containing immune serum showing trapping of antibody at the basement membrane (stained with fluorescein conjugated anti-rabbit Ig). x 740.

Fig. 8.6 A section from an explant cultured in medium containing IgG showing a colony of Candida growing between filiform papillae of rabbit tongue. The fluorescence indicated that anti-candidal antibodies from the medium have complexed with the fungi (stained with fluorescein conjugated anti-rabbit Ig). x 600.
However some specific staining of the basement membrane was apparent in some sections of those tissues cultured with immune serum or IgG. Specimens which had been cultured in media containing non-immune serum showed little fluorescence of *C. albicans* within the tissue, compared with those specimens cultured in presence of immune serum or IgG. In the latter cases *C. albicans* colonies on the explant's surfaces, together with fungi present within the epithelium and connective tissue were clearly fluorescent, indicating that antibodies present within the media had combined with antigens on the surface of the fungi (figs. 8.5, 8.6, 8.7).

### 8.4 DISCUSSION

The use of sera as nutrient media in tissue culture systems is well established. In the maintenance of epithelium *in vitro* some workers have encouraged its use as the major constituent of the medium; Sarkany *et al.* (1972) found at least 50% serum essential for culturing adult human skin. More common is the use of serum as a supplement for defined media. Cobb (1974) used 10% serum in maintaining fetal rat palate, while Scalleta and MacCullum (1972) used 10-20% for adult human mucosa. Thus in the present experiment the use of 20% rabbit serum in the maintenance of rabbit mucosa would not be expected to have deleterious effects on tissue survival or on its resistance to infection and in fact no morphological difference was seen between explants maintained in defined media alone or with serum added.

It is not proposed to discuss in great detail the methods used in the preparation of the immune serum and the tests for the antibody estimations as they follow standard immunological practice. However a few points are worth mentioning; in the original studies of Murray and Buckley (1966) 10 antigenic determinants were demonstrated on the surface of the yeast cell of *C. albicans* while between 68 and 78 have now been demonstrated by crossed immunoelectrophoresis (Axelsen, 1976). Hasenclever and Mitchell (1961) showed that *C. albicans* strains can be divided into two types of which type A has one more major cell wall
Fig. 8.7  Dark ground illumination reveals the presence of C. albicans within the tissues (a), while ultraviolet light demonstrates that antifungal antibodies have conjugated with the hyphae (b) in those explants maintained in media containing immune serum or IgG (stained with fluorescent anti-rabbit Ig).  x 2,200.
antigen than type B. In the present studies the same strain of *C. albicans* was used both in the preparation of immune sera and to infect the explants, to ensure consistency in the range of precipitating antibodies. Quantitative and qualitative differences in antigenic composition between the two growth phases of *C. albicans* have also been demonstrated and a specific mycelial antigen found (Evans et al., 1973) which was, in fact, a cytoplasmic component. The role of cytoplasmic antigens in superficial candidosis seems open to question, and it is possible that cell wall antigens predominate in the immune response to *C. albicans*. In the present studies antigen was prepared from both growth forms to ensure antibodies were raised to all available antigenic determinants.

The immunodiffusion test confirmed the presence of anti-candidal antibodies in immune serum although the precipitation lines were somewhat weak. The principle antigenic constituent of the fungal cell wall is mannann, which is soluble in water. However sonic disruption of cells, as used in these studies, produces complex antigen extracts which are also water soluble, and these, according to Taschdjian et al. (1964) are satisfactory reagents for gel precipitation tests. The concentration of extracts used for serological testing of *C. albicans* is extremely important as the precipitating complexes formed with cell wall mannann antigens are readily soluble in excess antibody or antigen (Faux et al., 1975); this may explain the weak reaction in the present studies. However, Faux et al. (1975) showed that two different sized antigen wells, as used in present tests, increased the number of positive reactions.

The presence of anti-candidal antibodies in the immune serum was confirmed by immunofluorescence, while immunoelectrophoresis demonstrated the presence of IgG in the extracted serum fraction and when subsequently diluted with culture medium. The complement fixation test showed that significant titres of anti-candidal antibodies were present in vitro. However despite this no alteration in the behaviour of *C. albicans* in the culture system was observed histologically. There are conflicting reports in the literature about the effect of serum on the behaviour of *C. albicans* in organ culture systems. Blank et al. (1959) cultured pieces
of excised fungal skin lesions and suggested that dermatophytes were prevented from invading the 'living' epidermis and dermis by the fungistatic effect of serum in the medium, and decreasing the concentration of serum did allow deeper invasion. *C. albicans* was mentioned by Blank et al. (1959) in these studies but no definitive results were given for the species. Pemberton and Turner (1973), on the other hand, found no inhibition of candidal growth in gingival epithelium cultured in 100% fresh human serum.

Non-specific factors inhibiting *C. albicans* which have been demonstrated in serum (see section 2.7.2) include transferrin (King et al., 1975) and the Candida clumping factor (Chilgren et al., 1967). The activity of transferrin would not be expected to affect the fungus *in vitro* as dilution of serum by buffered salt solution impairs its activity (King et al., 1975), while the amount of iron present within the system together with the carry over of iron with the large *Candida* inoculum would saturate any unbound transferrin leaving plenty of iron available as an essential growth factor for the fungi (Barlow et al., 1974).

Louria et al. (1972) demonstrated that the fungal clumping factor was still active in a 1 in 10 dilution of serum and neither penicillin or hydrocortisone affected the activity, however, antibodies to *C. albicans* were found to inhibit the activity with titres of 1:80 to 1:160. In the present studies no difference was seen in the ability of the immune and non-immune sera to control invasion by the fungi, and it appears that the clumping factor cannot be influencing the *in vitro* situation.

The immunofluorescence studies satisfactorily confirmed the presence of anti-candidal antibodies in the immune serum and the extracted IgG and demonstrated the distribution of candidal antigens in the tissues *in vitro*. *C. albicans* is autofluorescent (Berliner & Ryan, 1975) which may account for some of the staining observed in specimens reacted with non-immune sera. However the autofluorescence is reported to be restricted to the vacuole of the cell, except in dead organisms, and Berliner and Ryan (1975) state that this does not interfere with interpretation of
immunofluorescence which is predominantly associated with the cell wall. The cell wall fluorescence of organisms treated with non-immune serum may indicate the presence of some antibodies to non-specific "yeasts" in this serum which cross react with Candida antigens. Jones and Stewart-Tull (1975) have demonstrated cross reactivity between C. albicans cell wall antiserum and the cell wall of the common yeast Saccharomyces cerevisiae. It would seem likely that antibodies to a variety of yeast cell antigens existing in the natural environment of the rabbit are present in rabbit sera.

In those sections treated with immune sera, the staining of diffuse extracellular material associated with the brightly fluorescent cell wall of C. albicans may indicate the presence of soluble antigenic components which diffuse from the cell wall of Candida into the surrounding tissues. A role for such substances in the pathogenesis of candidosis has been suggested (Jones & Russell, 1973(a); Sohnle et al., 1976(a)) by which they may diffuse through the epithelium to the connective tissue and there meet with mediators of the inflammatory reaction.

The direct immunofluorescence test demonstrated the distribution of the anti-candidal antibodies in those tissues maintained in media containing serum. The test is not as specific as the indirect method, and a certain amount of non-specific background staining is often found. There is also some confusion as to the presence of antibodies within normal epithelial cells of the oral mucosa and Brandtzaeg (1975) suggested that serum proteins often diffuse into epithelial cells and become bound in the cytoplasm while Dabelsteen (personal communication) claimed this is not a common finding and that immunoglobulins are only present under abnormal conditions, for example when an inflammatory reaction allows leakage of serum proteins into the epithelium. The staining of the basement membrane seen in some tissues which had been maintained with immune serum or IgG may reflect trapping of antibody in this region, a similar immunofluorescence of complement deposits was demonstrated by Sohnle and Kirkpatrick (1977) in cutaneous candidosis in rodents.
In the explants which had been incubated in immune serum fluorescence of *C. albicans* demonstrated that anti-candidal antibodies had complexed onto fungi both on the surface and within the tissues. Whether the antigen-antibody association had formed before the hyphae had penetrated the epithelial cells or whether antibodies were able to penetrate the epithelium and thus contact the fungi within the tissues cannot be ascertained. Nevertheless the presence of anti-candidal antibodies complexed with the fungal cells did not prevent invasion of epithelium or connective tissue by *C. albicans*.

It seems that there is no obvious involvement for either the non-specific inhibitory factors or the specific immune ones incorporated in the in vitro model of mucosal candidosis. It could be expected that in vivo candidal antibodies act in conjunction with other factors not present in vitro and since starting this study a number of workers have demonstrated that restriction of fungal penetration is associated with a complement mediated inflammatory reaction (Ray & Weupper, 1976(a) & (b); Sohnle et al., 1976 (a) & (b)).

8.5 **CONCLUSIONS**

It has been shown that the addition of immune (anti-candidal) serum, non-immune serum or anti-candidal IgG to the in vitro system did not prevent the deep candidal invasion of mucosa seen in this model. Other systemic factors such as complement and inflammatory cells may be involved in the restriction of penetration in vivo.
CHAPTER 9

GENERAL DISCUSSION

9.1 INTRODUCTION
9.2 THE PROBLEM OF INVASION AND TISSUE RESPONSE
9.3 THE PATHOGENESIS OF THE LESION
9.4 CONCLUSIONS AND FURTHER WORK
9.1 INTRODUCTION

*C. albicans* is one of the small group of microorganisms including viruses, rickettsia, some bacteria and other pathogenic fungi which become intracellular parasites in man. Although a considerable knowledge of the methods by which many of these organisms invade and parasitise host cells has been obtained these organisms vary so widely in structure and behaviour that few similarities can exist in their relationship with host cells (Moulder, 1974; Mims, 1976). *C. albicans* is one organism whose behaviour has not yet been clearly established.

In both animals and humans, investigations of the mechanisms of oral epithelial invasion by *C. albicans* are hindered by the problems of obtaining satisfactory material for the study of the sequential events of infection. The difficulties include the problems of making a definitive clinical diagnosis of oral infection in animals and the delay in diagnosis in humans unless ethically questionable biopsies are taken. In the work described in this thesis the investigation of the host-parasite relationship in candidosis has been facilitated by the development of an *in vitro* model which provides abundant material for a sequential study of invasion. The model also allows evaluation of several of the factors which are thought to influence fungal infection of mucosa, including both local parameters such as variations in the epithelial surface, and factors of systemic origin such as serum components.

The first part of the investigation consisted of an evaluation of the model system while the later part involved ultrastructural, cytochemical and immunological studies of the host-parasite relationship established between *C. albicans* and the oral mucosa *in vitro*. Many of the findings arising from these studies have been discussed in the individual chapters. In this general discussion it is proposed to briefly review the results of the evaluation of the model system in the light of the succeeding studies and then to consider how the information obtained using this system relates to the existing knowledge of the pathogenesis of superficial candidosis.
The use of in vitro models with which to study the pathogenesis of disease will always be subject to scrutiny as to their reliability and their relevance to conditions in vivo. The present model depends upon the successful maintenance of neonatal epithelium in culture. From an examination of the histology of the epithelium in vitro (Chapters 3 and 4) and from an evaluation of its resistance to invasion by species of Candida less pathogenic than C. albicans (Chapter 4) it appears that the integrity of the epithelium was maintained in vitro. This finding was supported by subsequent studies of the fine structure (Chapter 6) and cytochemistry (Chapter 7) of the tissues, which showed that the epithelium maintained a similar ultrastructure and showed a similar localization of enzyme activity to epithelia in vivo. Hill has recently evaluated, in detail, various aspects of the culture system including qualitative and quantitative studies on the epithelial tissues up to 28 days in culture at both the light and electron microscopic levels and compared them to tissues in vivo (Hill, 1976, 1978; Hill & Miles, 1976, 1978). The present work, using similar methods of culture to those of Hill, is in agreement with his findings.

While this evidence supports neonatal oral mucosa in culture as a satisfactory host tissue in which to examine fungal invasion, the model also depends on the authentic behaviour of C. albicans in the system. This was evaluated in a comparison of the activity of Candida species of differing pathogenicity which established that Candida in vitro behaves as it does in vivo (Chapter 4). Ultrastructural and histochemical studies confirmed the similarity of the behavior of the fungi in the two situations. The fine structure of C. albicans and of the host-parasite relationship in the cultured tissues were similar to the relationship in vivo while evaluation by cytochemical techniques revealed that the distribution in the fungal cell of the hydrolytic enzymes, acid phosphatase and phospholipase A was generally in accord with biochemical and cytochemical reports in the literature (see section 7.5). In recent years several models of cutaneous candidosis in rodents have been proposed in which the development of infection by a number of Candida
species and the rate of fungal invasion of keratinized epidermis was similar to that seen in the mucosa in vitro (Ray and Weupper, 1976(a); Sohnle et al., 1976(b)). The evidence gained from the present studies and from the literature supports the current model of oral candidal infection as a satisfactory system in which to examine the relationship established between C. albicans and oral epithelium.

9.2 THE PROBLEM OF INVASION AND TISSUE RESPONSE

In the introduction to this thesis a number of questions concerning the host-parasite relationship in superficial candidosis were raised. These were related to the mode of invasion of the host cell by the fungus, the affects of the parasite on the host cells and tissue, and the manner in which fungal penetration of the epithelium is limited in vivo. In discussing how and to what extent the present studies have provided answers to these questions it is logical to first consider the mechanism of invasion of C. albicans.

It has been suggested that enzymes secreted by colonies of C. albicans growing on the tissue surface have a widespread effect on the underlying tissues, which is a prerequisite of lesion formation (Wain et al., 1976). Price and Cawson (1977) suggest more specifically that it may be a high concentration of lysolethycin which is the effective enzyme. In the present studies there was little evidence to support the role of generalized enzyme activity in cell penetration. In the histological studies of invasion by different Candida species there were degenerative changes in both epithelial and connective tissues in close contact with large fungal colonies which could be attributed to toxic products of the fungi (see section 2.6.3). However this behaviour did not always coincide with invasion. The ultrastructural investigations revealed no widespread damage of cell membranes, and breakdown of membranes associated with invasion of fungi was localized to the site of penetration. These findings suggest that the role of widespread enzyme activity in cellular invasion by C. albicans is secondary although as Wain et al. (1976) have
suggested it may play a more significant role in the formation of a clinical lesion. This will be discussed later.

Intracellular parasites can be either taken up by the host cell in a phagocytic process or the parasite can force its way in by expending its own energy in the effort (Moulder, 1974). Some bacteria have been shown to absorb to the cell surface and enter the cytoplasm directly after inducing a local breakdown in the plasma membrane (Mims, 1976). It appears from in vivo studies (Montes and Wilborn, 1968; Cawson and Rajasingham, 1972; Mohammed, 1975) and from the present studies that a similar invasive process is employed by C. albicans. The majority of workers have supported the hypothesis that the breakdown of the host cell membranes is an enzymatic process. In the present studies, the evidence for a specific role of phospholipase A in invasion was not as convincing as that proposed in recent studies by Pugh and Cawson (1977). However these workers used the chick chorio-allantoic membrane to study candidal infection and this may account for the discrepancies with the present results. The epithelium of the chorio-allantoic membrane is of a respiratory type which differs in structure from oral epithelium, which is primarily a protective surface covering (Cawson, 1972; Meyer et al., 1975). In particular the thickening of the superficial cell membranes of both keratinized (Squier, 1971) and non-keratinized epithelia (Frithiof, 1970; Squier, 1977), which has been claimed to be rich in disulphide compounds (Jesson, 1973), may offer resistance to the invading fungi. This could mean that hydrolytic enzymes other than phospholipase might play a significant role in mucosal invasion. The association of C. albicans with the keratinized epithelium of the skin has fostered the suggestion that a keratinase-type enzyme is produced by the fungus (Kapica & Blank, 1957; Montes & Wilborn, 1968); such an enzyme might also contribute to breakdown of the host cell membrane. Kapica and Blank (1957) examined the growth of C. albicans using keratin as the sole source of nitrogen, but found little substantive evidence for the presence of a keratinase which could be active in the rapid invasion of a keratinized epithelium.
Fig. 9.1 Diagrammatic representation of dermatophytic invasion of hair cortex by mechanical and enzymatic mechanisms (based upon English, 1963).
Extensive studies of the decomposition of keratin by dermatophytes have been undertaken and although it has been claimed that a specific protease or keratinase is involved (Yu et al., 1968, 1969), the majority of workers assume that keratin is first denatured by the fungi in order to render it amenable to less specific proteases (Kunert, 1972). It has been suggested that this denaturation is itself an enzymatic process (Chattaway et al., 1963) or occurs by cleavage of cystine bonds by sulphite produced during the growth of the fungi on cystine containing medium (Kunert, 1962). A number of proteases have been isolated from strains of C. albicans (Chattaway et al., 1971; Staib, 1965; Budtz-Jorgensen, personal communication) and it seems possible that similar processes may be involved in the invasion of the thickened squamous membranes and in the digestion of keratin, by C. albicans.

It appears that in fungi in general secretion of enzymes takes place from the growing apical tip (Chang & Trevitick, 1974) and from the present studies it is clear that the enzymes in C. albicans are localized to the small area of penetration; onward growth of the hyphae then appears to bring about a close contact between the epithelial cell cytoplasm and fungal cell wall.

Besides the apparent enzyme activity the distortion of both epithelial cell plasma membranes and nuclear membranes suggests a role for mechanical pressure in the invasive mechanism of C. albicans in the cultured mucosa. That mechanical force plays a part in fungal invasion would not be a surprising phenomenon as penetration by pressure is well known in fungal plant pathogens and English (1963) reported evidence for combined enzymatic and mechanical processes acting during the invasion of keratin (from human nail and hair) by a number of dermatophyte species. Some of the species English studied possessed specialised adaptations of their mycelia, the apical regions of which appeared to be able to enzymatically digest the surrounding keratin while pressure was exerted at this point allowing penetration of the tissues (see fig. 9.1).
Adhesive and cohesive forces bind fungus to host cell surface.

Minimal enzymatic digestion of host cell tissue maintains a tight seal between the host cell membrane and fungal cell wall.

Anchorage of hypha in surface squames.

Distortion of cell membrane.

Loss of cell cytoplasm.

Enzymatic digestion and pressure applied here.

**Fig. 9.2** Diagrammatic representation of the invasion of epithelium by *C. albicans.*
Implicit in the exertion of a force in one direction is an equal component in the opposite direction; English (1963) emphasized the important role of the anchorage gained from the parent mycelium, without which penetration of tissue did not occur. *C. albicans* lacks such a supporting mycelium such as that of the dermatophytes however Waid (1972) reports that fungal hyphae are in some way bound to their substrata at their contiguous surfaces and such forces as chemical adhesion, which may be brought about by mucilage production (Dickinson, 1960) or cohesion, where, for example, water molecules exist between a moist surface and hyphae, may play an important role in anchorage. Kimura and Pearsall (1978) have recently reported the adherence of *C. albicans* to buccal epithelial cells and in the present studies the fibrillar structure of the outer cell wall of *C. albicans* and its variation during the invasive process could suggest that this structure plays a role in the anchorage of *Candida* both on and within the epithelial cells.

Thus it could be proposed that the invasion of epithelium by *C. albicans* involves the adhesion of the hypha to the epithelial cell surface, the synthesis of extracellular enzymes from the apical tip of the hypha and subsequent digestion of the host cell membrane. Growth into the epithelial cell is accompanied by the establishment of close contact between epithelial cell membrane and cytoplasm and the post-apical hyphal walls. This would provide anchorage for the fungal cell so that onward growth could take place, facilitated not only by enzymatic digestion but also by mechanical pressure. However it could be suggested that mechanical pressure or enzymatic digestion alone play a limited role in invasion as evidenced by the cytochemistry, and an important feature is 'wedging' of the hypha against the cell membrane so that growth does not result in the fungus ejecting itself from the cell (see fig. 9.2).
In terms of the epithelial 'reaction' to the parasites two points arise; the apparent lack of response of cells in the superficial cell layers, which is also seen in vivo, and the phagocytic activity of the deeper epithelial layers. The ability of the epithelial cells to phagocyte material is supported by a number of findings. Wolff and Schreiner (1969) observed that foreign protein introduced into the epidermis was taken up rapidly by epithelial cells, and Mottaz et al. (1971) found that after stripping of the surface layers of the epidermis it was customary to see cell remnants in phagocytic vacuoles within the deeper epithelial cells. There is also evidence of phagocytosis of horseradish peroxidase (Squier, 1973) and thorotrast (Hayward, 1976) by oral epithelium. As proposed earlier (Chapter 7) enclosure of the invading fungi by phagocytic membranes may represent an attempt to exclude the parasites from the cells. The membranous response occurs at and below the level to which the fungi penetrate in vivo, so this mechanism may contribute to the exclusion of the fungi from the deeper epithelial cell layers in superficial candidosis.

The major difference between invasion of mucosa in vitro and in vivo is in the depth of fungal penetration. The present studies suggest that the deep fungal invasion seen in the cultured tissues is not due to changes within the host tissue itself, for the epithelial integrity appears to be retained (Chapter 4) and the relationship established between C. albicans and the superficial epithelial cell layers was similar to that seen in vivo. Thus it is proposed that the deep fungal invasion in the model is due to the absence of certain factors, probably of systemic origin from the in vitro system. In an attempt to define these factors it was found that the addition of immune (anti-candidal) serum, non-immune serum or anti-candidal IgG to the culture system failed to influence fungal behaviour (Chapter 8). This would suggest that the limited penetration in vivo is not solely dependent upon non-specific immune factors or anti-candida antibodies alone.
9.3 **PATHOGENESIS OF THE LESION**

A number of studies (Sohnle et al., 1976(a) & (b); Sohnle & Kirkpatrick, 1976; Ray & Weupper, 1976(b)) have recently shown that *C. albicans* can activate complement so releasing chemotactic factors and initiating an inflammatory response. Cutler (1977) has cited evidence that *C. albicans* itself produces factors chemotactic to polymorphonuclear neutrophils. It has been proposed (Ray & Weupper, 1976(a); Sohnle et al., 1976(a)) that the presence of inflammatory cells with their anti-fungal activity prevents the fungi from invading deeply. The ensuing inflammatory response is also followed by an increased rate of epithelial turnover with consequent desquamation of the infected squames, as was demonstrated in cutaneous candidosis in the rodent (Sohnle et al., 1976(b)). In the model of Sohnle et al. the response of the immune host differed, in that the inflammatory infiltrate was predominantly mononuclear and the amount of gross scaling of the squames was greatly increased. It has been suggested that the increased rate of epithelial turnover would rid the epithelium of the parasites (Sohnle et al., 1976(b)), especially in cases of minimal fungal invasion, for as rapidly as fungi entered the surface cells they would be lost. The turnover of epithelium in culture is significantly greater than in vivo (Hill, 1976). Despite this increase, fungal penetration of epithelium occurred, the hyphae penetrating at a considerable rate (0.3 µ/min.). Thus it seems that other factors, such as the presence of inflammatory cells, must restrict the invasion of the fungi, perhaps by killing the hyphae so they can be shed with the exfoliated squames.

It is interesting to note that the level to which the fungi penetrate is that at which the superficial epithelial intercellular permeability barrier begins (see section 2.3). This barrier extends through the surface layers and limits the penetration of water soluble substances both into the epithelium from the surface and outwards across the epithelium from the deeper tissues (Squier & Johnson, 1975). Soluble polysaccharide antigens from the fungal cell wall are the most chemotactic
fractions of *C. albicans* towards polymorphs (Davies & Denning, 1973) and it has been suggested that such substances diffuse through the epithelium from the fungi in the superficial layers (Sohnle *et al.*, 1976(b); Jones & Russell, 1973(a)). It is possible that only when the fungi have penetrated into the epithelium beneath the permeability barrier are these factors able to stimulate the inflammatory response. In support of this hypothesis Ray and Weupper (1976(a)) commented that deeper penetration of the epidermis took place in the first 24 hours of experimental infection of rodents, before an inflammatory response was evident, and subsequently the fungi assumed their more superficial location. One may question the relevance of an extracellular permeability barrier to intracellular parasites; however the substances diffusing from the fungi during passage of hyphae between cells are likely to penetrate through the intercellular spaces of the epithelium to reach the basement membrane.

Wain *et al.* (1976) suggested that the concentration of enzymes released from fungi on the surface of tissues is critical to lesion formation. As mentioned earlier in this discussion little ultrastructural evidence has been found in the present studies to support any mechanism involving direct effects of the enzymes on the epithelial cells. It might be suggested that the enzymes diffuse through the epithelium and provoke an inflammatory reaction in the underlying tissues but the intercellular permeability barrier would in all likelihood prevent diffusion of enzymes across the undisturbed epithelium (Squier & Johnson, 1975). However once the inflammatory process has been initiated by fungal invasion beneath the permeability barrier, the resulting disturbances of the epithelium and basement membrane may allow the diffusion of such enzymes across the tissue, from fungi on the tissue surface and in superficial cell layers, which may play a role in maintaining the inflammatory response and promoting active candidosis.

The role of such enzymes may be particularly significant in the chronic atrophic candidal infection associated with the wearing of acrylic dentures. This lesion appears to differ in histopathology from other forms of superficial candidosis in that fungi are not seen to be
invading the atrophic epithelium (Budtz-Jörgensen, 1974). Candida is found to colonize the fitting surface of the denture in contact with the tissues (Budtz-Jörgensen, 1974; Allison & Douglas, 1973) and it has been proposed that a delayed hypersensitivity reaction to the fungi or their metabolites is the significant factor in the pathogenesis of the lesion (Budtz-Jörgensen, 1974). It would seem likely that the fungi do in fact invade the epithelial cells but that these are rapidly lost from the atrophic epithelium. Candida hyphae invading epithelial cells attached to the fitting surface of a denture has been demonstrated in the scanning electron microscope (Douglas & Allison in Walker, 1975). It could also be proposed that disturbances of the permeability barrier in the lesion would allow diffusion of toxins or enzymes across the epithelium from yeast colonies in contact with the mucosa, so that the enzymes would play a predominant role in the maintenance of inflammation and hypersensitivity in the underlying tissues.

The ease with which C. albicans is able to invade the epithelial and connective tissues in vitro would suggest that once given the opportunity to colonize the surface of the oral tissues in vivo the fungus would have little difficulty invading the superficial epithelial cells. However as C. albicans is such a frequent member of the oral flora this would appear in contradiction to the number of clinical infections encountered. It may be that two factors are involved in the pathogenesis of superficial candidosis, firstly the fungal invasion of epithelium and secondly the initiation of a clinically defined lesion, the first event not inevitably leading to the second. It is possible that the occasional commensal organism in the oral cavity could readily invade the epithelium, the resultant minimal inflammatory response being all that is required to remove the organism from the epithelium and this would only represent a subclinical infection. A clinically defined lesion would result only when other factors influence the relationship, such as those shown to predispose to candidosis (see section 2.5). Thus invasion by large numbers of fungi as, for example, when antibiotic therapy allows overgrowth of Candida would result in a significant inflammatory response and a clinical lesion.
Invasion of a dystrophic epithelium by lesser numbers of fungi might result in persistant infection since the epithelium would fail to respond sufficiently to stimuli to increase turnover and rid the tissue of the parasites. In cases of immunodeficiency, whether involving acute inflammatory cells or lymphocytes which mediate cellular immunity, it appears that the inflammatory response is merely able to keep the parasites at bay and not rid the tissue of them altogether, the presence of the parasite provoking a chronic inflammatory lesion in the tissues. Similarly it appears that the virulence of fungal strains may affect the clinical response. Ray and Weupper (1976(a)) found that non-pathogenic species failed to stimulate a clinically defined lesion and it would seem that strains of *C. albicans* might also vary in this ability. Cutler (1977) has recently shown that the ability to attract polymorphs chemotactically differs with the virulence of the strain of *C. albicans*.

9.6 CONCLUSIONS AND FURTHER WORK

An *in vivo* model of candidal infection of oral mucosa has been shown to be a satisfactory system in which to study the development of the host-parasite relationship in superficial candidosis. These studies indicate that invasion by *Candida* species depends upon both the pathogenicity of the species and upon the degree of keratinization of the tissues, non-keratinized epithelium being more susceptible to invasion by less pathogenic species of *Candida*. *C. albicans* appears well adapted to rapidly invading oral epithelial tissues, and it appears that the mechanism of invasion involves both enzymatic and mechanical processes. As an intracellular parasite, *C. albicans* appears in the short term to cause little damage to superficial cells thus supporting the hypothesis that lesion formation in superficial candidosis is not related directly to damage to the superficial epithelial cells but to diffusion of antagonistic substances from the fungi through the epithelium to the deeper tissues. *C. albicans* is able to invade and proliferate in all layers of the epithelium in culture, which suggests that the restricted penetration of epithelium seen *in vivo* is not solely due to factors inherent in the epithelium,
While identifying many features of the host-parasite relationship, the present studies have also raised a number of questions which might form the basis of future work. It is possible that the outer cell wall of \textit{C. albicans} has a significant role in the pathogenesis of superficial candidosis. In the process of cellular invasion the outer coating may play a part in the adhesion of the fungus to the cells and form a matrix for extracellular enzyme activity. At the same time particles of this outer coat may diffuse away from the hyphae, as was suggested by the present ultrastructural and immunofluorescence studies and, by diffusing through the epithelium initiate an inflammatory response. The labelling of antigenic components of the cell wall at the ultrastructural level by immunocytochemistry might serve to demonstrate the location of such antigenic particles within the epithelium and support (or refute) a role for such particles in the pathogenesis of superficial candidosis.

If such behaviour was demonstrated it would be of interest to investigate whether the less pathogenic species of \textit{Candida} and strains of \textit{C. albicans} possess an outer cell wall with similar properties. The finding of Ray and Weupper (1976(a)) that invasion of superficial epithelium by less pathogenic species of \textit{Candida} failed to illicit lesion formation might suggest that either such antagonistic factors are not present, or if they do exist, fail to diffuse from the fungi or to stimulate an inflammatory response.

It has also been suggested in the present study that the epithelial permeability barrier influences the depth of fungal invasion \textit{in vivo} by preventing anti-candidal factors from reaching fungi in the superficial epithelial cell layers so that the penetration of fungi beneath the barrier must occur before the inflammatory response is initiated. It is hoped to investigate this possibility by examining the relationship of the permeability barrier to \textit{Candida} in biopsies of superficial candidosis.

By such investigations it would be hoped to establish how \textit{C. albicans}, a parasite in the superficial epithelial cell layers, provokes a response in the underlying tissues,
APPENDIX

1. The source, identification and maintenance of the *Candida* species.

2. Methods used in the immunological investigations:
   1. Preparation of antigen
   2. Extraction of IgG
   3. Immunofluorescent staining

3. Acknowledgements
APPENDIX I

The source and maintenance of the Candida species

The following Candida species were obtained from the Mycological Reference Laboratory at the London School of Hygiene and Tropical Medicine.

*C. tropicalis* 3111
*C. krusei* 3100
*C. parapsilosis* 3104
*C. guilliermondii* 3090
*C. albicans* strains 3091A (sero-type A), 3118c (sero-type B), which were originally isolated from the oral cavity, and 3153.

*C. albicans* strain 'T' was isolated from a chronic lesion on the tongue of a patient in the London Hospital, Whitechapel.

The strains were stored as freeze dried samples and during experiments were maintained on Sabouraud's dextrose agar (Oxoid Ltd., London) at 37°C and subcultured every 4 days.

The identification of the species was confirmed by the standard mycological tests reported by Mary D. English (1974, 'Identifying Yeasts', Med. Lab. Tec. 31:327). *C. albicans* was identified by its morphological growth forms including the development of germ-tubes on incubation in serum (serum germ-tube test) and by the development of chlamydospores on corn-meal agar. The identification of the remaining species was confirmed by morphological growth forms and the carbohydrate fermentation test.
APPENDIX 2

1. Preparation of antigen
   Antigen (a)

   A 24 hour growth of C. albicans 3153 on Sabouraud's agar (Oxoid Ltd., London) was washed in sterile normal saline, killed in 1.5% formalin, rewashed and suspended in saline. After using a haemocytometer to count the number of cells per ml., the suspension was diluted to give 5 x 10^5 organisms per ml.

   Antigen (b)

   The preparation was similar to that for antigen (a) except that C. albicans was grown in normal tissue culture media (T.C.M. as in experiment 1.) as Y-forms and M-forms, and were harvested by centrifugation. The final dilution was adjusted to give approximately 3.5 x 10^6 organisms per ml.

   Antigen (c)

   A 24 hour growth of C. albicans in T.C.M. was washed in saline, resuspended in 0.5 ml. of saline and maintained at 60°C. for 3 hours. The suspension was ultrasonicated for 10 minutes and stored at -20°C.

2. Extraction of IgG

   10 mls. of immune rabbit serum was dialysed against ethylene diamine/acetic acid buffer for 3 days at +4°C.

   5.0 gms. of Q.A.E. sephadex A.50 was equilibrated with 0.1 M ethylene diamine/acetic acid buffer ph 7.0 for 3 days at +4°C. The sephadex from which air had been evacuated at room temperature was then poured into a chromatography column (Pharmacia Fine Chemicals, Sweden).

   The dialysed serum was poured onto the column, the bed volume of the column (buffer) was discarded and the ensuing protein fractions (IgG) collected and stored at +4°C. 0.1 M acetate buffer pH 4.0 was poured onto the column at +4°C. to remove the remaining protein fractions.
3. Immunofluorescent Staining

a) Stain for the presence of anti-candidal antibodies in serum

The procedure involves the following steps:

i) *C. albicans* 3153 was grown overnight in tissue culture media.

ii) Smears were prepared on teflon coated slides (P.T.F.E. multispot slides Hendley Co., London), dried and flame fixed.

iii) Smears were stained with undiluted, 1:5, 1:10 or 1:20 dilutions of immune serum, non-immune serum or IgG from the immune serum for 20 mins. at room temperature in a moist environment.

iv) Washed for 20 mins. in phosphate buffered saline* (P.B.S.).

v) Stained with 1:8 fluorescein conjugated sheep anti-rabbit immunoglobulin (Wellcome Reags. Ltd., Kent).

vi) Washed for 20 mins. in P.B.S.

vii) Rinsed in distilled water.

viii) Dried.

*Phosphate buffered saline (P.B.S.)*

\[
\begin{align*}
\text{NaCl} & \quad \text{10.5 gms.} \\
\text{Na}_2\text{HPO}_4 & \quad \text{1.3375 gms.} \\
\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} & \quad \text{0.69 gms.} \\
\text{H}_2\text{O} & \quad \text{1000 ml. at pH 7.12}
\end{align*}
\]

b) Stain for the presence of *Candida* antigens in infected explants

i) 5µ frozen sections of tissue were cut.

ii) Sections were either washed for 20 mins. in P.B.S. or left as unwashed controls.

iii) Stained with 1:10 solution of IgG extracted from the immune serum for 20 mins.

iv) Washed with P.B.S. for 20 mins.

v) Stained with 1:8 fluorescein conjugated sheep anti-rabbit immunoglobulin for 20 mins.

vi) Washed with P.B.S. for 20 mins.
vii) Rinsed in distilled water.
viii) Mounted in glycerine and water.
ix) A further group of control sections (both washed and unwashed) were stained only with the fluorescent and anti-rabbit immunoglobulin, and washed and mounted.

c) Stain for the presence of anti-candidal antibodies in infected explants maintained in tissue culture media containing sera
i) 5μ frozen sections of tissue were cut.
ii) Washed in P.B.S. for 20 mins.
iii) Stained with 1:10 lgG, immune serum or non-immune serum for 20 mins.
iv) Washed in P.B.S. for 20 mins.
v) Stained with 1:8 fluorescein conjugated sheep anti-rabbit immunoglobulin for 20 mins.
vi) Washed with P.B.S. for 20 mins.
vii) Rinsed in distilled water.
viii) Mounted in glycerine and water.
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REFERENCES

The names of the periodicals are abbreviated according to the National Library of Medicine N.I.H., U.S.A. INDEX MEDICUS. 1976.


PEMBERTON, J. & TURNER, E.P. (1973) An organ culture model to study
82 in J. Dent. Res. 52:948.


Sabouraudia 15:179.

PUGH, D. & CAWSON, P.A. (1975) The cytochemical localization of phospho-

PUGH, D. & CAWSON, R.A. (1977) The cytochemical localization of phospho-
lipase in C. albicans infecting the chick chorio-allantoic membrane.
Sabouraudia 15:29.

RAY, T.L. & WEUPPER, K.D. (1976(a)) Experimental cutaneous candidiasis

RAY, T.L. & WEUPPER, K.D. (1976(b)) Activation of the alternative
(properdin) pathway of complement by C. albicans and related species.

Microbiol. Scand. 78:421.

REYNOLDS, E.S. (1963) The use of lead citrate at high pH as an electron

leukoplaikias: a histological and exfoliative cyto logical study.

ROONEY, M.L. & MOENS, P.B. (1973) Nuclear divisions at meiosis in the


PAPERS PRESENTED IN SUPPORT OF THE THESIS
THE INFECTION OF RAT TONGUE MUCOSA IN VITRO
WITH FIVE SPECIES OF CANDIDA

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PLATES XXI AND XXII

The pathogenesis of mucosal candidiasis has aroused much interest in recent years and several studies have revealed the morphological relationship between Candida albicans and the host epithelium in experimental infections of animal mucosa (Taschdjian, Reiss and Kozinn, 1960; Jones and Adams, 1970; Jones and Russell, 1973) and in human oral candidiasis (Cawson and Rajasingham, 1972). While such studies have demonstrated the host-parasite relationship of established fungal infection, investigations into the mechanisms of fungal invasion require a sequential examination of fungal entry into the tissues, which would prove tedious in animals and impracticable in human subjects.

Some reports would seem to encourage the use of organ-culture systems for studying candidal invasion. Blank et al. (1959) examined the pathogenesis of dermatophyte invasion of skin in culture, while more recently Pemberton and Turner (1973) investigated the experimental infection of human gingival mucosa in vitro with C. albicans. In both of these studies, similarities were demonstrated in the invasive behaviour of the fungi in vitro and in vivo. It therefore appears that organ cultures might be useful in investigating active invasion of oral epithelium by Candida. They might also provide an opportunity to study the host-parasite relationship in the absence of systemic factors, and the effect that differences in epithelial keratinisation might have on the course of fungal invasion.

In such studies it is important to establish that the tissue in culture is not merely acting as a passive growth medium in which any fungal strain, irrespective of its pathogenicity, could proliferate. To this end a comparison has been made of the activities in an organ-culture system of five species of Candida reported to have different pathogenicities (Hurley and Stanley, 1969); all have on occasion been implicated in human candidiasis (Hurley, 1966). The tissue employed in the model system was the dorsal tongue mucosa of neonatal rats, a tissue which Miles (1974) has successfully grown in culture for up to 10 days and which has also been shown to be susceptible to infection with C. albicans in vivo (Jones and Adams, 1970).
MATERIALS AND METHODS

The organ-culture system

Tissues were obtained from decapitated Sprague-Dawley rats aged 1-3 days. Explants about 2 mm square were taken from the dorsal surface of the tongue as described by Miles (1974), washed initially with Tyrode’s solution containing streptomycin 100 µg per ml and penicillin 200 units per ml, and then with nutrient medium. A chemically defined nutrient medium was used, based on Weymouth’s 75/21 medium (Wellcome Reagents Ltd) modified after Melcher and Hodges (1968) and incorporating ferrous sulphate 0.45 µg per ml, streptomycin 100 µg per ml, penicillin 200 units per ml, hydrocortisone sodium succinate 1 µg per ml and ascorbic acid 300 µg per ml. The tissues were supported on small pieces of Millipore filter type HA (pore size 0.45 µm) resting on stainless steel grids of the Trowell pattern (Trowell, 1954). These were placed in small plastic culture dishes and sufficient nutrient medium was introduced to reach the platform of the grids (about 1.5 ml). Cultures were then held in plastic petri dishes containing saline-moistened filter paper to maintain a humid environment. The petri dishes were stacked in a McIntosh and Fieldes jar and incubated at 37°C with a gas phase of 50% O₂, 5% CO₂ and 45% N₂ for the times indicated below.

Fungal organisms and preparation of inoculum

The strains of Candida used were obtained from the Mycological Reference Laboratory (London School of Hygiene and Tropical Medicine) and were as follows: C. albicans strains 3091A (serotype A) and 3118C (serotype B); C. tropicalis strain 3111; C. krusei strain 3100; C. parapsilosis strain 3104; C. guilliermondii strain 3090. The identification of the species was confirmed in our laboratory by sugar-fermentation tests. The isolates were stored as freeze-dried samples: during experimentation they were maintained on Sabouraud’s dextrose agar. To minimise the number of organisms used to infect the explants, a fluid micro-inoculum, containing only a few viable yeast cells, was prepared in 1% peptone water (Oxoid Ltd, London) from fresh 24-h cultures grown at 37°C. The optical density of the suspension was read in a spectrophotometer (S.P. 500, Pye Unicam Ltd) at a 400 nm wavelength, and the number of cells per microlitre of fluid was calculated from a standard reference curve, constructed by plotting the optical densities of different dilutions of a yeast suspension against viable counts obtained by the method of Miles, Misra and Irwin (1938). The yeast suspensions could thus be diluted to give the required number of viable cells per microlitre of fluid.

Experimental infection with C. albicans

The initial experiments investigated the behaviour of C. albicans in the culture system. Twenty-one explants were set up and 14 were inoculated approximately 4 h later with 5–15 yeast cells of C. albicans in 1 µl of fluid by means of 1-µl Microcap pipettes (Drummond Scientific App. USA), the remaining seven explants acting as uninfected controls. After inoculation, the original maintenance conditions were resumed and the tissues were harvested at intervals of 2 h between hours 18 and 30, and again at 39 h.

Experimental infection with various Candida strains

Fifty explants were set up and inoculated after either 4 h or 20 h as above with about 30 viable yeast cells of one of the following strains: C. tropicalis (13 explants); C. krusei (11 explants); C. parapsilosis (7 explants); C. guilliermondii (7 explants); C. albicans, strain 3091A (5 explants); C. albicans, strain 3118C (7 explants). The explants were then incubated under the original maintenance conditions until they were harvested at 30, 37 or 45 h after inoculation.

Histological preparation of tissues

Specimens were fixed in neutral formal saline solution and routinely processed for light microscopy, the sections being serially sectioned at 5 µm and stained with periodic-acid-Schiff
INFECTION OF RAT TONGUE MUCOSA WITH CANDIDA

reagent to demonstrate the presence of Candida, and counterstained with haematoxylin or tartrazine.

RESULTS

Assessment of uninfected control explants

The oral mucosa from the dorsal surface of neonatal rat tongue consisted of a stratified squamous, orthokeratinising epithelium that was organised into discrete, filiform papillae showing the dual pattern of keratinisation (Farbman, 1970). These papillae were supported by a cellular connective tissue, beneath which areas of muscle fibres could be seen. Generally this structure was well maintained in vitro, as described by Miles (1974), although tissues at the edges of the explants naturally underwent some disorganisation, and slight migration of the epithelium over the exposed connective tissues occurred. Otherwise stratification of the epithelium appeared normal and mitotic figures could be observed in the basal epithelial layers at all culture periods. An accumulation of keratin squames on the surface of the explants was noticeable even after short periods of culture; this was due to the lack of abrasive removal that normally takes place in vivo. The maintenance of connective-tissue structure was more difficult to assess by light microscopy and variation in appearance of the tissues could be noticed, including occasional areas of patchy acellularity in some specimens. However this did not seem to have any deleterious effects on the overlying epithelial cells.

Assessment of infection with C. albicans

In the experiments in which inocula containing about 10 yeast cells were employed, all of the inoculated explants showed evidence of infection, and colonies of C. albicans were seen to grow on top of the tissues. Blastospores (Y-phase) and mycelial forms (M-phase) of the fungus were present and by 18 h the M-phase could be seen penetrating the superficial keratin layer. As the incubation time increased, the penetration of the tissues progressed, so that by 24 h fungi could be seen in the granular layer of the epithelium, by 26 h in the prickle-cell layer, and by 28 h in the basal cell layer. After 30 h of incubation, infection of the explants had become extensive, and by 39 h it was very heavy, the hyphae passing through the epithelium and deeply penetrating the connective tissues (fig. 1). The predominant fungal form present within the tissues was filamentous (M-phase), although blastospores were also seen where tissue invasion was heavy. However, the Y-phase was never present alone in any of the cell layers.

An examination of the host tissues showed that superficial invasion of the epithelium had no deleterious histological effects on the deeper tissues. Where invasion by hyphae was more extensive, some disruption of the ordered structure of the epithelium was seen. Beneath an occasional surface fungal colony a flattening of the filiform papillae seemed to occur, although this was not a feature common to all infected areas, and some irregularities of papillary architecture were also visible in control explants. In regions of very extensive
invasion, as seen in specimens after 30 and 39 h, some thinning of the epithelium was visible.

Assessment of infection with Candida strains of different pathogenicities

Histological examination showed that Candida of all five species grew well under the conditions of culture and large numbers of fungi could usually be seen growing on and around the explants, perhaps reflecting the slightly larger inoculation dose and the increased post-inoculation culture-time used in these experiments. Besides fungal growth on top of the stratum corneum there was proliferation of organisms at the edges of the explant where the connective tissues were exposed, presumably because some of the inoculum had run over the edge of the tissues. This provided the fungi with the opportunity of invading the connective tissue directly.

The general pattern of invasion by these species is summarised diagrammatically in fig. 2. Explants inoculated with either strain of C. albicans showed a pattern of epithelial invasion from the surface similar to that in the previous experiment. Penetration at the edges of the explant into the connective tissue was also rapid, the fungal hyphae growing upwards through the tissues so that
by 37 h penetration of all the layers of the epithelium had also occurred by fungi entering via the connective tissue. At 45 h the explanted tissues were completely overrun by C. albicans.

In the culture systems infected with C. tropicalis both Y-phase and M-phase were present. Heavy growth of the fungi took place on top of the epithelium but on no occasion did penetration of the stratum corneum and invasion of the deeper epithelial cell layers occur from this direction (fig. 3). However, proliferation of C. tropicalis from the edges of the explant occurred with striking M-phase invasion of the connective tissues. This enabled the fungus to penetrate the basement membrane and lower nucleated cells of the epithelium from below, although no penetration of the keratinised layers of the epithelium from this direction was seen. Thus, by all appearances C. tropicalis lacked the ability to penetrate the intact stratum corneum either from above or below.

In the tissue infected with C. krusei the fungus grew in the Y- and M-phases but generally showed less invasiveness than C. tropicalis. Again, despite heavy growth of the fungi on the epithelial surface, no penetration of the stratum corneum was seen. Growth into the connective tissues at the explant edges was less extensive than with the former species, and only in a few explants did C. krusei reach and invade the nucleated cells of the epithelium (fig. 4).

Proliferation of C. parapsilosis and C. guilliermondii took place almost entirely in the Y-phase and only occasional short pseudohyphae were produced, mainly by C. parapsilosis. Active invasion of the epithelium or the connective tissue was not evident with either species (fig. 5). Despite heavy surface growth no penetration of the stratum corneum was seen, but there was sometimes flattening of the lingual papillae and possibly atrophy of the epithelium beneath the surface colonies, similar to that seen with C. albicans. Although only limited invasion of the connective tissue by these species occurred there seemed to be some degradation of the connective tissues in contact with colonies of blastospores at the explant edges.

**DISCUSSION**

The five species of Candida used in these experiments seemed to show a gradation of invasive activities in oral mucosa in vitro. C. albicans was undoubtedly the most invasive, being able to penetrate all layers of the epithelium and connective tissue, while at the other extreme C. guilliermondii seemed unable to invade actively any of the mucosal tissues within the duration of the experiment. This spectrum of invasiveness in vitro reflects the pathogenicity of the fungi demonstrated in living animals (Hurley, 1966) and in kidney-cell-culture systems (Hurley and Stanley, 1969). The latter workers proposed that specific aggressive mechanisms are possessed to a high degree by M-phase-producing species such as C. albicans and C. tropicalis and only to a low degree by species such as C. guilliermondii in which the Y-phase predominates. This hypothesis is clearly supported in the present studies since only C. albicans was able to penetrate the barrier of the stratum corneum and invade the rest of the tissues.
Fig. 1.—Rat tongue mucosa in vitro invaded by *C. albicans* showing blastospores proliferating on the surface of the epithelium and hyphae penetrating through the epithelial layers to the connective tissue. Periodic acid Schiff (PAS) and haematoxylin. × 360.

Fig. 3.—Surface colony of *C. tropicalis*. No invasion of the epithelium is occurring. PAS and haematoxylin. × 360.
Fig. 4.—C. krusei proliferating at the side of an explant. The hyphal form is seen invading the connective tissue and penetrating the basement membrane to enter the deeper nucleated cells of the epithelium. No penetration of the stratum corneum is observed. PAS and tartrazine. × 140.

Fig. 5.—A large colony of C. guillermondii growing alongside an explant. No invasion of the tissue is apparent. PAS and tartrazine. × 140.
Apart from invasion of the tissues, other pathogenic effects were common to all the fungal species used. These seemed to be attributable to the action of fungal metabolites within the culture system. A flattening of the lingual papillae and atrophic changes of the epithelium were often associated with heavy surface accumulation of fungi. It is interesting to note that in experimental candidal stomatitis in the monkey (Budtz-Jorgensen, 1971) atrophy of the epithelium was reported, although no intra-epithelial fungal penetration was described.

The behaviour of *C. albicans* observed in organ culture was similar to that previously reported in cultured gingival mucosa (Pemberton and Turner, 1973). The different lag times before fungal invasion starts probably reflect variations in technique and the nature of the explanted tissues. It has been shown ultra-structurally that *C. albicans* is an intracellular parasite *in vivo* (Montes and Wilborn, 1968; Cawson and Rajasingham, 1972) and this is also true *in vitro* (Howlett, 1974). Histological studies on infected cell cultures have suggested that hyphae of other *Candida* species are also able to enter nucleated epithelial cells (Hurley and Stanley, 1969) and this appeared to be the case with *C. tropicalis* and *C. krusei*, the M-phases of which penetrated the Malpighian cells in the cultured mucosa. Thus, the failure of these fungi to penetrate the stratum corneum is not indicative of a general inability to invade cells but suggests that the keratinised squames offer particular resistance to the less pathogenic *Candida* species. The pattern of progressive invasion seen here seems to indicate that the mucosal tissues *in vitro* are not merely an accumulation of cells serving as a passive growth medium for the fungi, but behave in many ways like tissue *in vivo*. This supports the view that the culture system is a useful experimental model in which to investigate the interaction between invading fungi and host epithelium.

One striking difference between in-vivo and in-vitro invasion of the oral mucosa by *C. albicans* is the deeper penetration of mucosal tissues seen in culture. Hyphae in mucocutaneous candidal lesions in man are confined to the superficial epithelial layers and such restricted penetration has also been reported in experimental infection of rat (Jones and Russell, 1973) and mouse mucosa (Taschdjian et al., 1960). It has been suggested that invasion is limited by local factors within the deeper nucleated epithelial cells which are not conducive to fungal growth (Taschdjian et al., 1960). While such local factors may have been lost *in vitro* it would seem more likely that the deep penetration reflects the absence of systemic components within the culture system, as suggested by Blank et al. (1959). It is interesting to note that an intercellular permeability barrier has been demonstrated in the superficial layers of the epithelium of both skin (Schreiner and Wolff, 1969) and oral mucosa (Squier, 1973). This barrier limits the penetration of substances both into the epithelium from the surface, and outwards across the epithelium from the deeper tissues. The level to which hyphae are seen to penetrate in mucocutaneous candidiasis seems to coincide with the lower border of this barrier. It is possible that systemic factors antagonistic to *Candida* are present beneath the barrier and discourage fungal penetration of the deeper epithelial layers, while
the absence of such factors in the superficial layers allows the fungus to colonise this region.

**SUMMARY**

Orthokeratinised mucosa from the dorsal surface of neonatal rat tongue was maintained in culture and then infected with *Candida albicans*, *C. tropicalis* *C. krusei*, *C. parapsilosis* or *C. guilliermondii* for up to 45 h. The five species showed varying abilities to invade the tissues, which appeared to reflect their different pathogenicities. *C. albicans* was the only species able to invade all the tissues present, including the stratum corneum. *C. tropicalis* and *C. krusei* were able to invade connective tissue and the deeper nucleated cells of the epithelium but failed to penetrate the keratin layer, while *C. parapsilosis* and *C. guilliermondii* showed only slight invasion of the connective tissue. The keratin layer of rat tongue mucosa thus appeared to act as a barrier to invasion of the epithelium by anything but virulent species of candidal fungi.

The results suggest that oral mucosa in vitro retains its structural integrity and that the tissues do not act solely as a passive growth medium through which any fungal strain might proliferate. It seems that this in-vitro system is representative of the in-vivo situation and forms a useful experimental model in which to investigate the host-fungal relationship in mucosal candidiasis.

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**REFERENCES**


Candidal Infection of the Oral Mucosa: An in vitro Model

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Fungi belonging to the genus *Candida* are feeble, opportunistic pathogens yet, despite this, *C. albicans* must be one of the commoner causes of oral mucosal disease. Although some 40% of the population carry the yeast as a normal mouth commensal (Bartels & Blechman 1962) there is an increasing awareness of various factors which can tip the balance of host resistance and allow this
ubiquitous fungus successfully to challenge the body’s defence mechanisms and cause disease. Candida-associated diseases vary from the relatively innocuous and common denture stomatitis to far more serious disseminated infections. The increasing incidence of candidiasis has aroused much interest in the pathogenesis of this condition and studies in recent years have done much to improve our understanding of the relationship between the fungus and its host. Clinical investigations have revealed factors which predispose the individual to candidal infections such as the use of steroid, antibiotic and immunosuppressive therapy (Walker 1975), while experimental studies in both animals (Hasenelever & Mitchell 1961, Jones & Russell 1973) and man (Maibach & Kligman 1962, Reboras et al. 1973) have examined the response of host tissues to candidal invasion. At the same time mycologists have studied the behaviour of the fungus alone with particular regard to its potential as a pathogen (Taschdjian & Kozinn 1961, Chataway et al. 1971).

One of the experimental approaches used in recent years in the investigation of mucocutaneous diseases, including candidiasis, has been that of establishing an in vitro model system. Chick chorio-allantoic membrane has been used in this way to assess the pathogenicity of candida species (Partridge 1959) and to study the hyperplastic response of an ectoderm to candidal invasion (Cawson 1973). The culture of human gingival tissue and its infection with C. albicans was proposed as a useful model by Pemberton & Turner (1973) but more recent studies on the maintenance of oral mucosa in vitro (Miles 1974, Hill 1976) suggest that neonatal animals would be a more reliable source of oral tissue for in vitro studies.

The study to be described here involved the infection of cultured animal oral tissues with candida species as a model system of mucosal candidiasis. The tissues were taken from neonatal animals and were from three different regions of the oral mucosa so as to reflect the spectrum of epithelial keratinization; thus the dorsal mucosa of rat tongue provided an orthokeratinized mucosa, the rabbit tongue a parakeratinized mucosa and the rabbit buccal mucosa a non-keratinized mucosal surface. Explants of epithelium and some supporting connective tissue 1–2 mm square were prepared and maintained by the method of Miles (1974), using a chemically defined nutrient medium. Within 24 hours the explants were inoculated with candida, either by a microinoculation technique which distributed 10–15 yeast cells on the tissue surface, or by means of a fine-gauge wire which provided a greater number of yeasts. The tissues were cultured for varying periods of time up to 40 hours and were then harvested and processed for light or electron microscopy.

The initial experiments established that the tissue in culture was not acting merely as a passive growth medium in which any fungal strain irrespective of its pathogenicity could proliferate. This was achieved by comparing the invasive activities in the in vitro system of five species of candida previously reported to have different pathogenicities (Hurley & Stanley 1969). The activity of the species was assessed by histological examination but in some cases superficial
invasion of the epithelium was difficult to discern so that confirmation of actual invasion was sought at an ultrastructural level using the scanning electron microscope. All the five species of candida selected—C. albicans, C. tropicalis, C. krusei, C. parapsilosis and C. guilliermondii—grew well under conditions of culture and large numbers of fungi could be seen growing on and around the explants. C. albicans was the only species able to invade all the tissues present in the various types of mucosa. The hyphal phase of C. albicans could be seen to penetrate the superficial keratin layer of rat tongue by 18 hours. As the incubation time increased the penetration of the tissues progressed so that by 28 hours the hyphae had penetrated the basal epithelial cells and basement membrane (Fig 1) and by 35 hours were deeply invading the connective tissue. C. tropicalis and C. krusei were unable to penetrate the keratin layer and invade tissues from the surface, but yeasts growing around the edge of the explants proliferated next to the exposed connective tissue and so invaded this tissue and reached the lower nucleated cells of the epithelium from below. C. parapsilosis and C. guilliermondii showed no active invasion of either epithelium or connective tissue. The keratin layer of rat tongue thus appears to act as a barrier to invasion by all but the virulent strains of candida (Howlett 1976).

Such an effective barrier was not present in the nonkeratinized epithelium of rabbit cheek mucosa. C. albicans again invaded all the tissues present and C. tropicalis was also able to penetrate from the surface as far as the middle layers of the epithelium (Fig 2) while C. krusei only entered the superficial layers during the period of culture and C. parapsilosis and C. guilliermondii did not invade at all. Confirmation of epithelial invasion was obtained with the scanning electron microscope by means of which hyphae of C. albicans were seen entering both keratinized and nonkeratinized mucosa (Fig 3) and hyphae of C. tropicalis and C. krusei could be seen invading the surface cells of nonkeratinized epithelium. The pattern of progressive invasion displayed by these species of candida seems to indicate that mucosal tissues in vitro are not merely acting as an accumulation of cells serving as a passive growth medium for the fungi, but behave in many ways like tissues in vivo. This finding supports the view that the culture system is a useful one in which to study the interaction between invading fungi and host epithelium, and the remaining portion of this paper presents some morphological aspects of the invasion of oral epithelium by C. albicans which is the major pathogen. The initial invasion of mucosa in vitro by C. albicans bears a close resemblance to the naturally occurring disease. Long pseudohyphae penetrate through the superficial layers of the epithelium, the yeast form being present within the tissues only when invasion is heavy. However, one striking difference from the behaviour of the fungus in vivo is the deeper penetration of epithelial tissues seen in culture. Hyphae in mucocutaneous candidiasis in humans and animals are usually confined to the superficial epithelial layers and it has been suggested that this is due to factors produced within the deeper epithelial layers which limit fungal growth (Taschdjian & Kozinn 1960). While such local factors may have been lost in vitro it would seem more likely that
the deep penetration reflects the absence of systemic components within the culture system. However, it is possible to add such factors as serum and soluble antibody fractions to the culture system, and recent experiments have evaluated the effect of adding both serum and isolated IgG fractions from rabbits immunized against *C. albicans*, as well as non-immune rabbit serum. The initial results suggest that these factors alone do not significantly alter the pattern of tissue invasion *in vitro* and it seems likely that complement and the cellular immune system also play an active role in the restriction of candidal invasion *in vivo* (Ray & Wuepper 1975).

Ultrastructural investigations have shown that fungal invasion of the superficial layers of the epithelium *in vitro* is similar to that described and illustrated *in vivo* by Cawson & Rajasingham (1972) and more recently by Mohamed (1975). As can be seen in Fig 4 fungal cells appeared scattered in both intracellular and extracellular locations among the loosely attached superficial squames, while deeper in the epithelium the hyphae were invariably intracellular. The presence of the candida within the superficial cells seldom...
Experimental Isografts of Odontogenic Tissues

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It is known that isografts of whole mammalian tooth germs exhibit continued differentiation and growth. However, the ectopic environment commonly promotes dysplastic and metaplastic changes within the graft, especially squamous metaplasia with cystic degeneration in the enamel organ. This latter change may provide an experimental model of the pathogenesis of dentigerous cysts, the rationale of which has recently been reviewed by Harris & Toller (1975).

The broad aim of the present investigations was to graft components of primate teeth and tooth germs at several stages of development and to examine their capacity for further growth and metaplasia. The teeth of young monkeys (Macaca fascicularis) were chosen because of their close similarity to the human dentition and because they are sufficiently large to be manipulated without sacrifice of the animal. The subcutaneous layer offers a large field for grafting but the identification of small grafts for harvesting is problematical. To facilitate relocation a modification was used of a method described by Lewis-Epstein (1964), in which granulomas induced by the subcutaneous implantation of expanded polyvinyl alcohol (PVA) sponge were used as a graft bed. Our pilot studies in rats (unpublished data) had shown that sponge granulomas supported continued growth of tooth germ isografts and that the large surface area of sponge matrix appeared selectively to encourage the proliferation of metaplastic epithelium.

**Methods**

A single graft bed consisted of a 1 cm cube of PVA sponge penetrated axially by a solid polyamide rod, 2 mm in diameter and about 8 mm in length which protruded slightly from one surface. The rod held patent a channel for the placement of the graft in the mature granuloma. Sets of cubes were implanted subcutaneously in the monkey's back; after 3-4 weeks the PVA sponge was completely infiltrated by granulation tissue and was easily palpable through the skin. At the time of grafting the granuloma was uncovered through a small incision, the rod located and removed (Fig 1), and the graft placed in the distal extremity of the channel. The rod was then shortened as necessary, gently replaced and the skin incision sutured. Sham instrumentation of