Early events in the invasion of skin, nail and hair by dermatophyte fungi

By

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DECLARATION

I hereby declare that this thesis embodies the results of my own original work, that it has been composed by myself and has not been submitted for consideration for any other degree in this or any other University.

Azer Rashid

Dedicated with gratitude to

my parents

in recognition for their

love, patience and tremendous support

"And of the knowledge, you have been given but a little"

THE QUR'AN

' 'Surat Al-Isra, Chap. 15, V.85' '

SUMMARY

The early events in the invasion of keratinized tissues (stratum corneum, nail and hair) by dermatophyte fungi were investigated by employing arthroconidia of two strains, one of *Trichophyton interdigitale* and one of *Trichophyton mentagrophytes.* New *in vitro* experimental model systems were employed to study the colonization of these tissues by dermatophyte fungi, including the germination and hyphal extension of such fungi, and their response to antifungal agents. The germination of arthroconidia and hyphal extension on different body surfaces (face, back of the hand, palm, leg and sole) was determined by using stripped sheets of stratum corneum from these areas. The ultrastructural relationship between arthroconidia and germ tubes while growing on the stratum corneum was investigated by scanning and transmission electron microscopy. The possibility of using this stratum corneum model for assessing antifungal drugs was explored by pre-exposing the corneocytes to therapeutic concentrations (6.2–100 mg/L) of terbinafine and assessing the effect on germination and hyphal extension of *T. interdigitale* arthroconidia. The morphological changes induced by terbinafine in the arthroconidia and germ tubes of *T. interdigitale* whilst growing on the stratum corneum were also observed.

The morphological transformation of dermatophyte arthroconidia on nails was studied by using distal nail clippings (finger, toe) and observing the process of nail invasion by germlings of *T. mentagrophytes* in the absence of added nutrients with scanning and transmission electron microscopy. This nail model was used to assess the antifungal activity of terbinafine against dermatophytes by pre-exposing the nails to low concentrations (0.001–10 mg/L) of terbinafine and then studying its effects on the germination and hyphal extension of *T. mentagrophytes* arthroconidia. At the same time observing morphological changes in the growth forms, growing on terbinafine-exposed nails with scanning and transmission electron microscopy.

A novel hair model was explored for detailed study of the invasion of hair and the effects of antifungal drugs on the invasion process by dermatophyte fungi. This was achieved observing the morphological transformation of *T. mentagrophytes* arthroconidia on dissected human terminal hair follicles and plucked anagen hair maintained in organ culture. These were then exposed to low concentrations (0.01–10 mg/L) of terbinafine and determining the effect on germination and hyphal extension and morphological changes induced in these growth forms. This whole process was studied with light microscopy and by scanning and transmission electron microscopy. Similarly, the human living skin equivalent was utilised to examine the establishment and growth of *T. mentagrophytes* arthroconidia, and to subsequently assess the effects of terbinafine. Germination and hyphal extension of arthroconidia was studied on the living skin equivalent. Low concentrations of terbinafine (0.01–10 mg/L) were incorporated in the model. The effect on fungal growth was examined histologically using haematoxylin and eosin – and periodic acid/schiffs (PAS) stained sections and by scanning electron microscopy.

Germination of *T. interdigitale* arthroconidia was observed on all body surfaces (face, back of hand, palm, leg and sole). 100% germination of arthroconidia occurred on face and back of hand and leg while 80–95% germination was seen on palm, leg and sole by 24h. Hyphal extension was observed on all the body surfaces. There was a direct relationship between percent germination and hyphal extension on face, back of the hand and leg and an inverse relationship on palm and sole. A mycelium was in place at most of these sites by 48h. Close adherence of arthroconidia and germlings was confirmed by scanning and transmission electron microscopy. A fibrillar–floccular material bridged the intercellular space between the arthroconidial wall and the surface of the corneocytes. A mixed picture of germinated and ungerminated arthroconidia and germ tubes of various lengths was seen, with the appearance of side branches and secondary germlings. Germ tubes and side branches were observed growing on and in the direction of corneocytes. It

appears that factors present in corneocytes are crucial for the establishment of dermatophyte infection on the stratum corneum.

Germination of arthroconidia was partially inhibited by therapeutic terbinafine concentrations though hyphal extension was totally inhibited on the body surfaces. Dose dependent morphological changes were observed in the arthroconidia and germ tubes. This was verified by SEM and TEM. Drug induced pores and craters were seen in arthroconidia while others were destroyed. Germ tubes showed inhibition of growth at extremely low concentrations of terbinafine. They were swollen and dilated with pores observed in the germ tubes and side branches which gradually enlarged. The surface of the germ tubes exhibited exfoliation with collapsed hyphae at higher concentrations of drug tested.

Adherence and germination of arthroconidia on the nails was observed. Germlings grew confluently on the surface of the nails. On SEM on gross examination penetration of the nail was observed at 24h with germlings penetrating through crevices on the ventral surface of the nail. By 4 days nails were fully covered with mycelial growth. At 2 weeks all of the layers of the nail were invaded. This was confirmed by transmission electron microscopy. Chains of arthroconidia were seen growing through well formed channels in the nail. Growth of fungal elements was seen to be mostly intercellular. Where nails had been pre-exposed to terbinafine, growth inhibition occurred at concentrations of 0.01-10 mg/L. At terbinafine concentrations of 1 mg/L and above no growth was seen. Morphological changes in arthroconidia and germ tubes growing on terbinafine-exposed nails included a roughened surface and pores in arthroconidia, with a broken mycelium, collapsed hyphae and unaffected areas on the nails.

Rapid germination of arthroconidia with very firm adherence to the hair surface was seen. At 24h on SEM there was evidence of penetration underneath cuticular cells and fungal elements were seen growing in the hair cortex by 6 days as revealed on transmission electron microscopy. On

toluidine sections fungal elements were seen to penetrate through all the hair layers and grew very well on the inner root sheath. Initially the cuticular cells seemed to act as a barrier to the invasion of the shaft but later on the whole shaft was invaded. With longer incubation most of the hair was digested and replaced by fungal mycelium. Where hair follicles were in the presence of terbinafine, germination was seen to commence on the surface of the follicle but inhibition of further hyphal extension was evident with areas of the follicle devoid of fungal growth, at drug concentrations of 0.01 and 0.1 mg/L. At a concentration of 1 mg/L and above there was no growth on the hair or any evidence of hair penetration. Scanning electron microscopy revealed damaged arthroconidia and hyphae which were dilated and swollen with club ends.

Arthroconidia adhered to, germinated and produced hyphal apices, side branches and mycelia by 72h on the living skin equivalent. The hyphae penetrated the skin equivalent, being observed in the dermal component. Hyphal penetration of the dermis does not occur *in vivo*, suggesting that *in vivo*, certain serum factors may limit dermatophyte growth to the stratum corneum. Where terbinafine was incorporated in the skin equivalent inhibition of fungal growth was seen at concentrations at and above 0.01 mg/L. At 1 mg/L no growth was seen , and no evidence of penetration of the dermis by hyphae was seen in the presence of terbinafine suggesting that this drug is somehow deposited in the dermis. On scanning electron microscopy dilated and collapsed hyphae with damaged arthroconidia were seen.

In summary this study contributed the following findings with regard to the early events involved in the pathogenesis of dermatophytosis in keratinized tissues and the dermatophyte–antifungal drug interaction in these tissues, also to the *in vitro* testing of antifungal drugs:– The pattern of growth of dermatophyte fungi on all the keratinized tissues have similarities: Adherence of arthroconidia to keratinized tissues appears to be the first event which is followed by rapid germination of arthroconidia on these tissues and penetration of the tissues by germlings, in between cells leading to deeper invasion, which

appears to be a combination of mechanical and chemical forces acting together, one augmenting the other. This process is established with the formation of arthroconidia in the tissues. This enhanced ability of dermatophytes to invade through the thickness of these tissues leads to the establishment of infection overcoming the proliferative and defensive capability of these tissues. From a therapeutic point of view any form of treatment for dermatophyte infection needs to be able to penetrate these keratinized tissues rapidly at fungicidal levels and be able to damage the arthroconidia, which are the main mediators of disease transmission. Terbinafine is not only deposited in the stratum corneum, epidermis, and dermis but also somehow incorporated in the other keratinized tissues such as nail and hair and acts as a barrier against further invasion of these tissues by dermatophyte fungi. Dormant arthroconidia appear to be more susceptible to terbinafine. Prophylaxis of ringworm in endemic areas with this drug may be highly effective since arthroconidia coming into contact with the keratinized tissues will be damaged and morphological transformation to tissue invasive hyphal form will be inhibited.

The assessment of antifungal agents is fraught with several difficulties. *In vitro* sensitivity tests for determining the activity of antifungal agents against filamentous fungi are inappropriate and often correlate poorly with *in vivo* efficacy. Experimental studies with animals are expensive, complicated and time consuming and do not always give reproducible results. Clinical trials with antifungal drugs are difficult to control because of lack of uniformity of naturally occurring lesions. Clinical evaluation of antimycotics by experimental infection in humans has ethical problems and like in experimental infection in animals, natural remission of disease may occur thus causing difficulty in detecting the therapeutic effect of the drug. To overcome all of these difficulties new experimental model systems for testing antifungal drugs have been described in this study. The advantage of these models is that they enable the study of antifungal activity on fungi in their natural environment, stratum corneum, nail

and hair. The findings of this study support their use in pre-clinical evaluation and testing of antifungal drugs.

Chapter 1

General Introduction

1:1 Dermatophytes

The dermatophytes belong to a broad group of fungi with pathogenic capacity in man and diverse animal species. They are keratinophilic agents, that is they only parasitize keratin or keratinized structures (cornified epidermis, hair, horn, nail and feathers) of humans and other animals (Rippon, 1988). In humans they cause dermatological lesions encompassed within the term dermatophytosis (tinea or ringworm infections). They are all moulds belonging to three genera of the fungi imperfecti: *Microsporum*, *Trichophyton* and *Epidermophyton*. Emmons (1934) defined three genera of dermatophytes according to the morphology of their macroconidia, which are fusiform with tapered ends with rough walls in *Microsporum*; cylindrical, club shaped and narrow with smooth walls in *Trichophyton*; and pear shaped or broadly club shaped with rough walls in *Epidermophyton*. As with a number of fungi the dermatophytes may exhibit two phases in their life cycle, and hence may be known by two different names, the anamorph (imperfect or asexual phase) which is the state isolated in the laboratory, and the teleomorph (perfect or sexual phase). Not all dermatophytes have been shown to have a perfect or sexual phase (Rippon, 1988).

The human infecting dermatophytes are also grouped according to the ecology and reservoir of the organism. Geophilic strains grow and thrive in soil where they may contaminate or infect the coats of animals especially small rodents and thus infect man through an intermediate animal host. Zoophilic dermatophyte species normally parasitize animals, however all the known zoophilic species occasionally cause infection in humans characteristically producing highly inflammatory lesions even to the extent of tissue destruction. There is a strong tendency for the lesions to heal spontaneously. Anthropophilic strains are primarily parasitic on humans. Strict anthropophilic species depend entirely on humans for their survival and reproduction. Such dermatophytes are widely distributed throughout the world, however certain

species are geographically restricted and endemic in particular areas of the world (Rippon, 1988).

The laboratory diagnosis of a dermatophyte infection is made by examination of material from the lesion (skin scraping, nail and hair clipping). In the tissues they are present as hyphae, which are regular in width, may branch and have cross walls (septa) which may be so frequent that the hyphae are divided into numerous segments called arthroconidia (arthrospores). All dermatophytes give this appearance in the skin and the actual species involved can only be determined by culture. Dermatophytes can be readily isolated on simple glucose peptone agar media and in most instances primary growth forms the distinctive microscopic and macroscopic characteristics by which they are classified (Richardson and Evans, 1989). It is the development of various structures from or on the hyphae for purpose of propagation or penetration which give the characteristic features associated with dermatophytes grown on culture media. Depending on the environment in which they grow, the dermatophytes produce two types of asexual propagules, saprophytic conidia or parasitic conidia. Under non-parasitic conditions they produce macroconidia and microconidia. While in a parasitic environment dermatophytes exclusively produce arthroconidia. These conidia possess the property of dormancy and are usually resistant to environmental conditions. They are important propagules of dermatophyte species and also mediators of infection (Hashimoto, 1991).

1:2 Dermatophytosis

The infection caused by dermatophyte fungi in the keratinized tissue such as hair, nails and stratum corneum of the skin is known as dermatophytosis. It is among the most prevalent mycotic infections in the world (Rippon, 1988). Its incidence is highest in hot, humid climates or in crowded environments where a high standard of hygiene is difficult to maintain. An important characteristic of the dermatophytes as parasites is their restriction to

the dead keratinized tissue. The reason that dermatophytes fail to invade the deeper layers of the skin is that they cannot survive in the presence of serum; only in the keratinized tissue are they protected from a serum inhibitory factor (Carlisle *et al.*, 1974).

The inflammatory response of ringworm infection involves the dermis and stratum malpighii of the epidermis, but the fungus itself is found growing only within the stratum corneum, within and around the fully keratinized hair and in the nail plate and keratinized nail bed. While all dermatophytes invade the stratum corneum, different species vary widely in their capacity to invade hair and nail. *Trichophyton rubrum* rarely invades the hair but frequently invades nail. *Epidermophyton floccosum* never invades the hair and only occasionally the nail. *T. mentagrophytes var.interdigitale* is commonly associated with tinea pedis (Rippon, 1988). The clinical appearance of the various forms of ringworm infection is largely dependent on the body sites affected. The lesion results as a combination of direct damage to the keratinized tissue by the fungus (mainly in hair and nail infections) and the inflammatory manifestations. Destruction and disorganisation of hair and nail occur with various degrees of intensity whenever they are involved. Various factors modify the basic type of lesion, including site of infection. Classically the lesions of ringworm infection are scaly, erythemato-vesicular with peripheral extension and central clearing, which may occur anywhere on the trunk and limbs. The fungal hyphae are present at the periphery of the lesion in the active or inflammatory border and the annular lesions usually described are thought to be due to increased epidermal turnover at the periphery of the lesion (Berk *et al.*, 1976).

Dermatophyte lesions cease to grow after they have attained a certain size and spontaneous resolution of these lesions is not uncommon, clinical symptoms often disappear even if left untreated (Jones *et al.*, 1974). This most probably occurs due to the development of cell mediated immunity. In some cases however, the lesions do not heal and the infection becomes chronic (Hay, 1982). Dermatophytosis is not a debilitating or life threatening infection

and serious invasive disease has been reported but only in patients who are immunosuppressed (Ahmed, 1982). It is transmissible from person to person directly by means of contact or indirectly via fo mites contaminated with infected skin scales or hairs and it can be acquired by humans from infected animals and by direct exposure to infected soil. Essentially all humans come into contact with dermatophyte fungi during their lives but only a small percentage of people manifest clinical symptoms of disease and some people therefore feel that dermatomycosis is not a contagious disease (Rippon, 1988).

1:3 Pathogenesis of dermatophytosis

1:3:1 Mediators of disease transmission in dermatophytosis

Dermatophytosis is unique among mycotic infections because of the communicable nature of the infection. There have been many outbreaks of dermatophytosis in schools, orphanages, chronic care institutions, dormitories and other places (Hashimoto, 1991). Disease transmission is mediated by two distinctly different types of propagules depending on the source of infection. (Fig 1:1). From saprophytic sources such as soil or shed scales of animals, the disease is transmitted to others by saprophytic conidia, macro- or micro-conidia, while from humans the disease is transmitted to others by parasitic conidia, arthroconidia (Hashimoto, 1991). All these conidia possess the property of dormancy and resistance (Hashimoto and Blumenthal, 1978; Hashimoto, 1991; Aljabre *et al.*, 1992b). No saprophytic conidia are produced by dermatophytes under going parasitization and possibly the mechanism controlling saprophytic conidiogenesis in the dermatophytes is suppressed under the state of parasitization (Hashimoto, 1991). Arthroconidia are the only infective conidia produced under parasitic conditions and are the natural pathogenic elements of the dermatophytes and the major mediator of disease transmission in dermatophytosis (Hashimoto and Blumenthal, 1977; Aljabre *et*

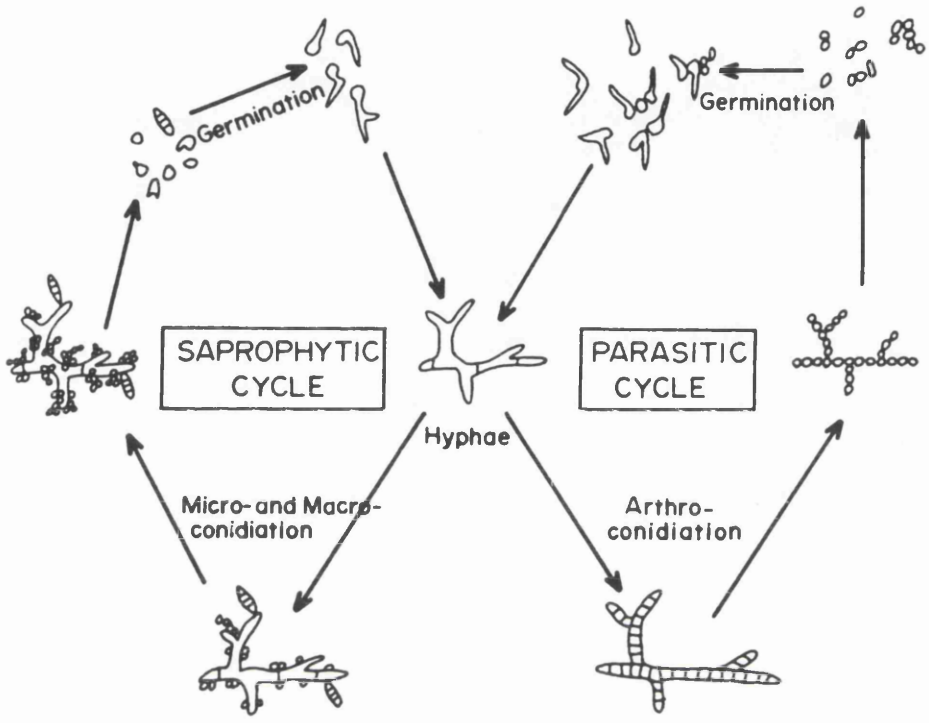


Figure 1:1 Asexual life cycle of *T. mentagrophytes*. (Adapted from Hashimoto, 1991)

al .,1992a) and the only form of conidia found in tissues (Gotz, 1959; Hashimoto, 1991). Kligman (1955) suggested that arthroconidia were the main source of transmission of dermatophytes from one person to another, being present or surviving in fallen hair, and loosened squames. It is further supported by the fact that arthroconidia are the only resistant morphologies produced by the dermatophytes during parasitization and that viable dermatophytes can be recovered from clinical specimens of skin, hair and nails stored for extended periods of time under adverse conditions (Dvorak *et al.*, 1968). Many surveys have shown that dermatophytes can be isolated from combs, brushes, the back of theatre seats, caps, bed linen, towels, undergarments and jockstraps. Locker rooms and shower floors, communal shower rooms are often contaminated with skin debris containing infective arthroconidia (Hashimoto, 1991).

Aljabre *et al.* (1992a) demonstrated that germination of arthroconidia and hyphal penetration were important factors in the pathogenicity of *Trichophyton.* Further convincing evidence that dermatophyte arthroconidia are infective and capable of transmitting infection was obtained by experimental induction of tinea pedis in guinea pigs by inoculating *T. mentagrophytes* arthroconidia. The lesions produced were clinically and histopathologically similar to human infection (Fujita and Matsuyama, 1987).

The infectious arthroconidia are very resistant to environmental extremes and are able to survive up to 20 months in a viable state in skin and hair (Dvorak *et al.*, 1968). Therefore the original contact with the arthroconidia is usually indirect and adherence of infected hair or skin scale carrying the arthroconidia is the major way by which dermatophyte fungi are transmitted from one source to another (Richardson and Aljabre, 1993). The arthroconidia of *T. mentagrophytes* demonstrate an exogenous type of dormancy provided that the conditions are not lethal and Aljabre *et al.* (1992b) have shown that dormant arthroconidia can germinate when conditions become suitable. As arthroconidia are formed by fragmentation of hyphae these dermatophyte

spores are most suitable for the growth of dermatophytes in the stratum corneum, unlike macro- or micro-conidia which are formed laterally on the hyphae thus requiring a space for their expansive growth (Hashimoto, 1991). The precise mechanisms involved in the formation of arthroconidia in the tissues are not clear. King *et al.* (1976) suggested that arthroconidial formation in lesions may be stimulated by the diffusion of CO_2 through the skin. Allen and King (1978) demonstrated that occlusion of the skin rendered it more susceptible to dermatophyte infection by raising the carbon dioxide tension on the skin surface. Hashimoto (1991) suggested that elevated temperature, high humidity, high carbon dioxide tension and some stimulatory metabolites may all contribute to arthroconidiation of the dermatophytes in the skin.

In vitro formation of arthroconidia has been extensively studied (Bibel *et al.*, 1977; Emyanitoff and Hashimoto, 1979; Hashimoto, 1991, Fig 1:2). Emmons (1934) described arthroconidia in dermatophyte cultures grown *in vitro* though generally the formation of parasitic arthroconidia requires a living host. The most critical factor affecting arthroconidiogenesis in dermatophytes appears to be the incubation temperature. Temperatures between 32°C and 39°C (37°C optimal) strongly favour arthroconidiation of *T. mentagrophytes* in Sabouraud dextrose broth (Emyanitoff and Hashimoto, 1979). Other factors which are important for arthroconidia formation in dermatophytes include medium pH, high humidity, CO_2 concentration and certain stimulatory metabolites (Hashimoto, 1991). *In vitro* conditions for arthroconidia formation bear some resemblance to factors present physiologically in the skin. It is possible that yet unidentified cutaneous factors may be involved with predisposing physiological conditions in arthroconidia formation *in vivo* (Richardson and Aljabre, 1993). It is possible that like in plants some form of chemical signal or topographical signal along with temperature signals may be involved. Fungi respond morphologically to a varied array of physical and chemical signals, including touch, light, pheromones and nutrients (Hoch and Staples, 1991). Signalling processes have been elucidated in mammalian cell

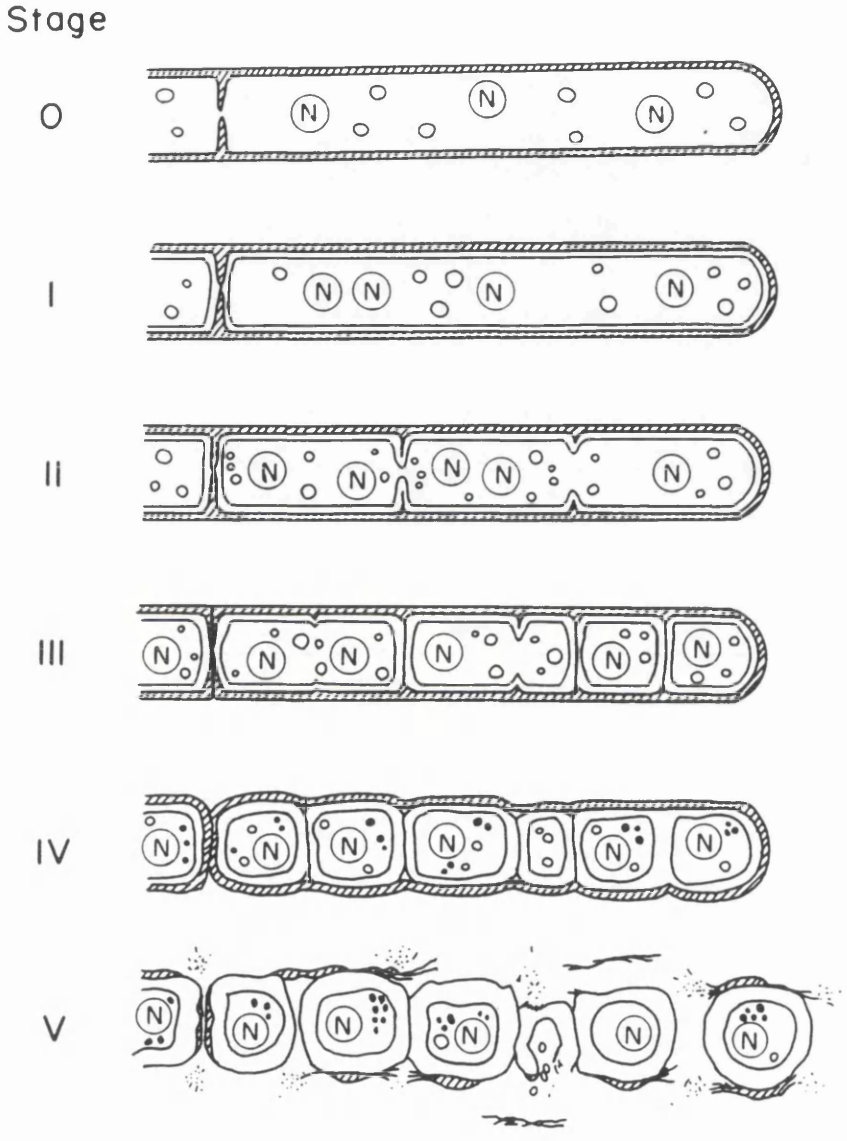


Figure 1:2 The different stages in the development of arthroconidia in *T. mentagrophytes.* The main conidial wall (inner wall) is clearly formed *de novo* during arthroconidiogenesis. The original hyphal wall partially disintegrates during arthroconidiogenesis and only its remnants remain on the surface of the mature arthroconidia. (Adapted from Hashimoto, 1991)

systems and the major signal transduction pathways are active in keratinocytes (Rosenbach and Czarnetzki, 1992). Cyclic AMP cascade is involved in the control of dormancy and induction of germination in fungal spores (Thevelein, 1984). The presence of transmembrane signalling systems and receptors in dermatophytes have not as yet been elucidated.

1:3:2 Mechanism of disease initiation in dermatophytosis

Dissemination of dermatophyte fungi depends on direct or indirect contact between infected and uninfected hosts. The first step in the infection process is colonization of the cornified surface of the stratum corneum. The initial contact between arthroconidia and stratum corneum seems to be the important event in the establishment and initiation of skin, hair and nail infection (Richardson and Aljabre, 1993). With the exception of white superficial onychomycosis in which the nail is invaded directly on its surface, nail and hair are infected from the adjacent stratum corneum (Richardson and Aljabre, 1993). Zurita and Hay (1987) and Aljabre *et al.* (1993) have demonstrated the interaction between dermatophyte arthroconidia and corneocytes, and the importance of close adherence in initiation of infection. Arthroconidia seem to be able to adhere to all body surfaces, face, back of hand, palm, leg and sole (Aljabre *et al.*, 1993). Adherence of arthroconidia to corneocytes occurs by 6 hours and increases with time. Aljabre *et al.* (1993) showed that the number of corneocytes with adherent arthroconidia and number of arthroconidia adhering per corneocyte increases with time. Experimental tinea pedis has been incited in guinea pigs with a relatively small number of arthroconidia (Fujita and Matsuyama, 1987). Once an arthroconidia has made contact with stratum corneum it becomes firmly adherent, possibly by some form of physical or chemical binding taking place between the arthroconidia and corneocyte. Ultrastructurally it has been observed that there is a very close contact between dermatophyte arthroconidia and corneocyte with the presence of a fibrillar-

flocullar material in the space between the arthroconidial outer wall and corneocyte membrane (Aljabre *et al.*, 1993; Rashid *et al.*, 1993). The nature and origin of this material, whether from the arthroconidial wall, the corneocyte or both is not clear. It has been observed that arthroconidia of dermatophytes undergo swelling before emergence of a germ tube, (Scott *et al.*, 1984) and the crenated surface of corneocytes flatten forming a hollow where arthroconidia adhere (Rashid *et al.*, 1993). The significance of these changes on arthroconidial adherence to corneocytes is not understood.

It is clear that arthroconidial adherence to human corneocytes is an early stage in colonization of the skin by dermatophytes. Adherence prevents arthroconidial detachment from host surfaces and enables them to remain on the stratum corneum and develop into the hyphal form and establish infection. Aljabre *et al.* (1992a) showed that germination of arthroconidia and hyphal penetration of stratum corneum are important factors in the pathogenicity of *T. mentagrophytes*. The arthroconidia must germinate very rapidly and penetrate the body surface otherwise they will be lost by continuous desquamation of epithelium (Richardson and Aljabre, 1993). Germination of arthroconidia occurs very rapidly, usually in 4–6 hours (Aljabre *et al.*, 1992a) and is the first step in the development of fungal mycelium from arthroconidia; by 7 days a well formed mycelium is observed on stratum corneum (Aljabre *et al.*, 1992a). The corneocytes express soluble factors which when added to isolated arthroconidia induce germination. Factors isolated and characterised from corneocytes of human skin have been shown to promote germination of arthroconidia (reviewed in Richardson and Aljabre, 1993). Moisture in the skin appears to break the dormancy of arthroconidia and encourage germination (Aljabre *et al.*, 1992b). Desquamated skin scales are normally dry, but if there is an increase in the moisture content as in a shower cubicle or swimming pool, the state of dormancy is broken and germination and penetration of host surface can then commence (Richardson, 1990). Arthroconidia invading in the presence of corneocytes demonstrate prolonged survival which suggests that

the stratum corneum confers some form of protection to arthroconidia and is epidemiologically significant as by increasing the longevity and thereby the infectivity of arthroconidia (Richardson, 1990).

The next phase of infection is the penetration of the stratum corneum by the invasive germlings. This process has been studied by stripping layers of stratum corneum from body surfaces with adhesives (Knight, 1973a), and then inoculating with arthroconidia (Aljabre *et al.*, 1992a). The penetration of the stratum corneum starts with the emergence of germ tubes from arthroconidia which are lying extracellularly. Germ tubes invade corneocytes and grow transversely and between the layers and through the thickness of the stratum corneum. The horizontal extension results in the clinically observable sign of peripheral expansion of lesion in dermatophytosis. Micro-colonies develop, and the developing hyphae disarticulate into arthroconidia completing the cycle (Aljabre *et al.*, 1992). The penetration of germ tubes into deeper layers of the stratum corneum has been seen in experimentally induced dermatophytosis (Fujita and Matsuyama, 1987). Intracellular location of dermatophytes has been seen (Miyazaki *et al.*, 1966). The mechanism by which germ tubes rupture the corneocyte membrane is not clear, although it is thought to be a combination of chemical and mechanical forces (Richardson and Aljabre, 1993).

Dermatophytes are known to produce enzymes capable of keratin digestion and keratinases have been detected in lesions of experimental dermatophytosis in guinea pigs using fluorescent antibody probes (Koga *et al.*, 1986).

Some of the extracellular hydrolytic enzymes produced by dermatophytes include proteases, lipases, phosphatases, nucleases and glucosidases. Among these proteolytic enzymes, keratinases, collagenase and elastase are most frequently implicated in the pathogenesis of dermatophytosis (Hashimoto, 1991). Some of these enzymes are involved in facilitating the penetration of germ tubes or hyphae into keratinous epidermal tissues, securing

nutrients for fungal growth and eliciting local inflammatory immune responses. The ability of dermatophytes to produce hydrolytic enzymes *in vivo* is an important virulence factor of dermatophytes. It is not yet clear if these enzymes play any significant role in disease initiation in dermatophytosis (Odds, 1991). From the pathogenic point of view, adherence of arthroconidia to corneocytes along with their germination and penetration of the stratum corneum can be regarded as mechanisms operative in the establishment of dermatophytosis.

1:4 Host defence mechanisms against dermatophytosis

The mechanisms that defend the host against dermatophyte infections can be arbitrarily divided into local defences such as the skin and systemic defences such as the non-specific and specific immune systems.

The skin is a tough, resistant, multi-layered structure whose main function is protection. The epidermis is constantly proliferating and hence corneocytes are shed from its surface. For dermatophytes to establish an infection the arthroconidia have to adhere and then rapidly germinate, and the growth of the fungus must keep pace with the rate of epidermopoiesis and exfoliation (Richardson and Aljabre, 1993). Increased susceptibility to dermatophyte infection of the palms occurs in patients with palmo - plantar keratoderma where there is excessive retention of stratum corneum (Nielsen, 1984). In psoriasis which is characterized by hyperproliferation of the epidermis, the incidence of dermatophyte infection has been found to be low (Fransson *et al.*, 1985). Dry intact stratum corneum acts as a barrier against infection (Richardson, 1991), and moist areas between toes in shoe wearing people and the groin in males are particularly susceptible. Macerated skin appears to provide an excellent environment for germination of infective arthroconidia (Richardson and Aljabre, 1993).

The stratum corneum is covered with a film made up of the products of keratinization (desquamated corneocytes), sweat and products of sebaceous

glands (surface lipids) and a unique microbial flora. Unsaturated fatty acids found in sebum inhibit some dermatophytes *in vitro*. Resistance of adult scalp to tinea capitis and the spontaneous clearing of tinea capitis in children at puberty at a time when greater quantities of sebum begin to be formed has been attributed to the presence of fatty acids. Other factors which are to be considered are sweat, which is thought to be inhibitory to the growth of dermatophytes, possibly because of vitamin K like substances (reviewed in Richardson and Aljabre, 1993).

Human serum has been reported to have a factor inhibitory to dermatophytes which is possibly involved in limiting the growth of the dermatophyte fungi to the stratum corneum (Carlisle *et al.*, 1974). King *et al.* (1975) identified this factor as being unsaturated transferrin. It inhibits growth of dermatophytes by binding to iron which organisms need for their growth. Transferrin in serum is thought to diffuse through the epidermis to the stratum corneum and inhibit fungal growth (Jones *et al.*, 1974). Dermatophytes grow poorly at 37°C and this may explain why deep infections with dermatophytes are almost unknown. Failure of immunity in persistent infections and its relationship with chronicity are not understood but there seems to be an association between atopy and chronic dermatophytosis (Jones, 1980). For example Kaaman (1985) found a decreased proportion of T-helper cells and increased number of T-suppressor cells in patients with chronic infections.

Antibody response occurs in the inflammatory and non-inflammatory type of infection. The cell mediated immune response as indicated by delayed type hypersensitivity occurs more readily in the inflammatory type of infection (Svejgaard, 1985). Antibodies appear to have no protective effect in dermatophytosis. Dermatophyte infections mostly occur in perfectly healthy people but can be more frequent and severe in immunodeficient patients. Other factors important in the susceptibility and resistance to infection are malnutrition where fungal infections are more common. Increased susceptibility to ringworm occurs in patients with Cushings syndrome and possibly diabetes.

There are age and sex related factors and similarly genetic and racial factors. There seems to be an increased incidence of dermatophytosis in the elderly. Tinea cruris (groin ringworm) is unheard of in females, tinea pedis, tinea cruris and tinea unguium are more common in males. Tinea capitis is more common in boys. Negroid skin seems to be relatively less susceptible to dermatophyte infection than caucasoid and in negroid skin there is less inflammation to infection than skin of other races. Tinea corporis caused by *T. rubrum* is particularly intractable in blacks. Susceptibility to tinea inbricata is inherited as an autosomal recessive characteristic (reviewed in Hay *et al.*, 1992).

Systematic immunological responses induced by dermatophytes are both antibody and cell mediated. The non-inflammatory system is the primary defence of the non-immune host because it does not require previous exposure to the invading organism for activation and can respond immediately to protect the host against an invading fungus. Acquired immuno-resistance mechanisms appear after adequate exposure to the organisms.

A very early event in the host reaction to invasion by dermatophytes includes infiltration of neutrophils and epidermal oedema (Ackerman, 1979) and to a lesser extent monocytes accumulate in the epidermal infiltrate probably in response to chemotactic components of the dermatophyte cells like C5a anaphylatoxin via the alternative complement pathway (Jones *et al.*, 1974). Dermatophytes are damaged by toxic oxidative products produced by neutrophils and are destroyed by neutrophils extracellularly. Dermatophyte arthroconidia do not need an opsonin for ingestion by neutrophils to take place and can be readily ingested by neutrophils (Richardson, 1990). The precise role played by the chemotactic epidermal cytokines particularly Interleukin 8 in the accumulation of neutrophils at the site of dermatophyte infections is not clear (Schroder, 1992).

From dermatophyte experimental infection studies in animals and man, and in natural human infections, T-cell responses such as the development of delayed hypersensitivity to dermatophyte antigens correlates not only with the

development of inflammation in ringworm, but also with spontaneous recovery. Chronic infections are associated with poor T-lymphocyte mediated immune response to specific fungal antigens. The ultimate establishment of dermatophytosis and the subsequent course of the infection are influenced by other host factors such as innate immune state, previous exposure to dermatophytes or inherent genetic predisposition.

1:5 Stratum corneum

The stratum corneum is a heterogeneous structure forming the outermost layer of the epidermis and comprising of layers of dead, keratinized, flattened, interdigitated epidermal cells (corneocytes). Functionally the stratum corneum is the most important part of the skin forming a tough and flexible membrane, the superficial aspect of which is constantly being shed as large clusters of squames. It serves as a barrier to the free passage of fluids and electrocytes, and protects against microbial and physical injury (Elias, 1989).

The stratum corneum is composed of corneocytes which are flattened, polyhedral cells with a convoluted surface and have lost their nuclei and cytoplasmic organelles. The cells are filled with the tonofilament matrix complex, surrounded by a thickened rigid and chemically resistant cornified cell envelope. The cells are bound together by modified desmosomes and with intercellular spaces filled with lipid-rich material, derived from membrane coating granules (Elias, 1989).

The lipids of the stratum corneum are concentrated largely in the extracellular space, and cement the structure together into a coherent membrane, principally consisting of triglycerides, free fatty acids, free sterols and ceramides, virtually devoid of phospholipids. This two-compartment model has been analyzed by means of a brick wall, resulting in the so-called "bricks and mortar model", where lipids form the "mortar" holding the keratinized "bricks" together (Elias, 1989).

Each skin region has its own structural and physiological peculiarities. Kligman (1964) classified the stratum corneum into two morphologically distinct forms, the horny layer over palms and soles which is thicker, so adapted for weight bearing and friction and the horny layer over the rest of the body adapted for flexibility, impermeability and fine sensory discrimination. The permeability of the skin, the so called epidermal "barrier" function largely resides in the stratum corneum, it is only a thin layer at its base, the stratum compactum, which actively retards water loss or the entrance of exogeneous materials (Ebling, 1992). The efficiency of the barrier differs between body sites. The scrotum is particularly permeable and the face, forehead and backs of the hands may be more permeable to water than the trunk, arms and legs. The palms are particularly impermeable to nearly all molecules except water (Ebling, 1992). Individual corneocytes are approximately 30μm in diameter and 1–2μm thick although variation occurs due to age, sex and site (Plewig and Marples, 1970). Marked regional variation in both the mean thickness and the mean number of cell layers has been documented for different regions of the body (Holbrook and Odland, 1974). There are various factors which favour the dermatophyte fungi to invade the stratum corneum: the stratum corneum is avascular with dead cells, it is well hydrated, is composed of various factors like amino acids, protein, lipids and carbohydrates which favour the growth of dermatophytes and anatomically certain areas favour the establishment of dermatophyte lesions (reviewed in Richardson and Aljabre, 1993).

1:6 Targets for antifungal drugs

The increasing incidence of fungal infections has greatly stimulated interest in the development of new antifungal drugs and in the study of their mechanisms of action. The dramatic increase in the incidence of fungal infections has been due to a combination of improved recognition and diagnosis of the mycoses as well as the existence of larger populations that are

immunocompromised. An increased incidence of fungal infections in an immunologically compromised group is the population afflicted with Acquired Immune Deficiency Syndrome (AIDS). Opportunistic fungal infections are the major life threatening complications of this disease (Drouhet and Dupont, 1990). Another problem is the emergence of strains of fungi that are resistant to marketed antifungal drugs (Macura, 1991).

The most prevalent fungal infections are cutaneous. For such superficial fungal infections the assessment of new antifungal compounds is necessary in order to produce effective and acceptable treatment (Rippon and Fromtling, 1993). The treatment of patients with impaired immune system calls for new antimycotics which are both fungicidal and as free as possible from side effects. These two considerations are relevant not only to the therapy of systemic mycoses but also to the topical and oral treatment of superficial mycoses if prompt healing and reduced relapse rates are to be achieved (Kerridge and Vanden Bossche, 1990). The ideal antifungal agent needs to have the following properties; broad spectrum of activity, fungicidal, no development of resistance during therapy, be available for oral and intravenous tissue administration, penetrate body fluids, achieve high levels in skin, nail and hair and have low toxicity (Kerridge and Vanden Bossche, 1990). Indeed a variety of antifungal drugs have been developed from the viewpoint of both therapeutic potency and minimal side effects. However, few agents are at present available for the satisfactory application to patients.

Antimycotic drugs interfere with the normal lifecycle of fungi by inhibiting normal functioning of one or several vital cellular entities. The effects of these agents are reflected in an altered pattern of growth, differentiation, transformation, ultrastructure and viability of the fungus. A number of potential targets for novel antifungal agents have been discovered whose mode of action can be explained by interference at these targets (Fig 1:3). The molecular target sites and subcellular target organelles for antimycotic action have been identified for most antifungal drugs (Borgers, 1988).

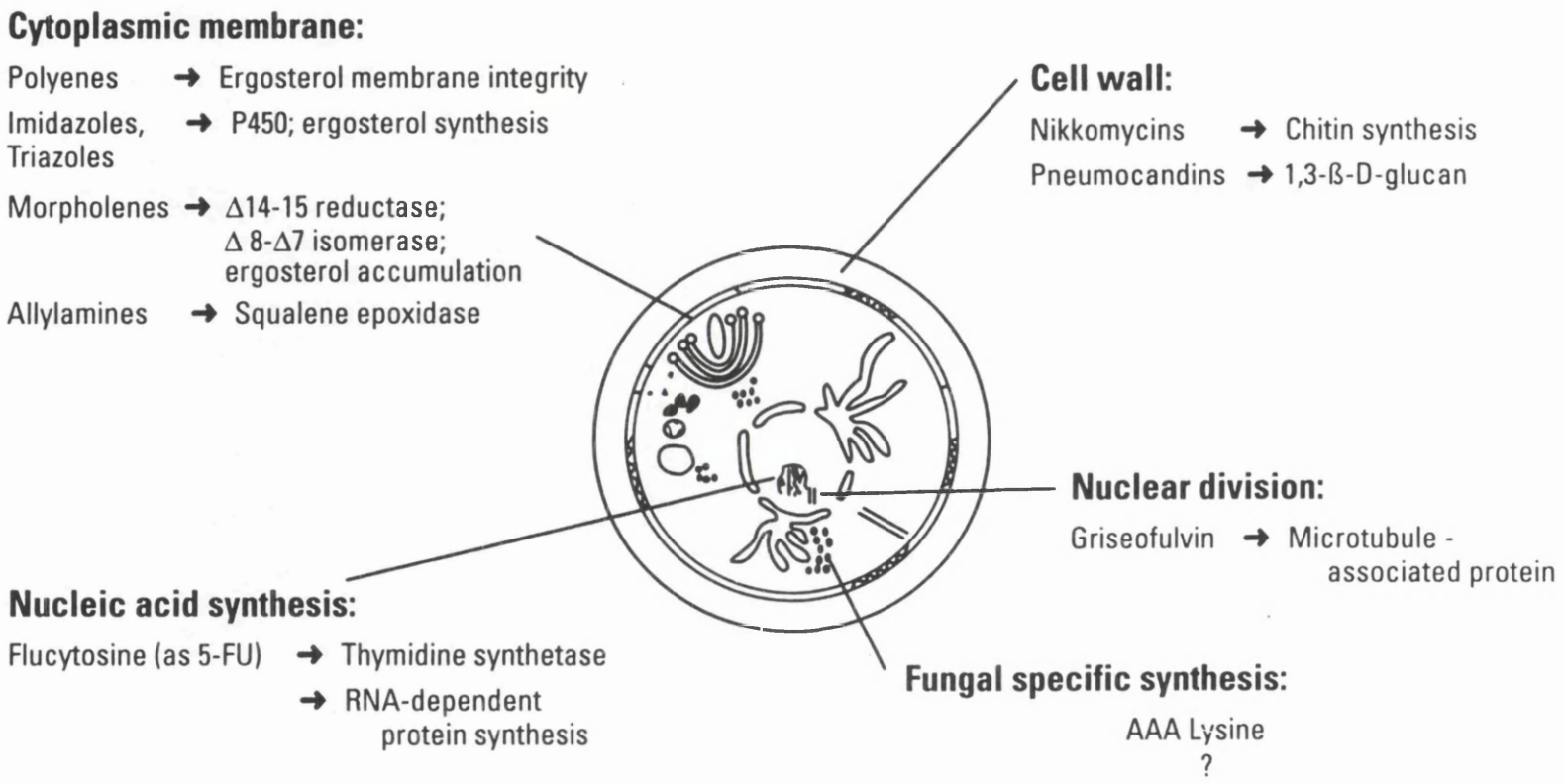


Figure 1:3 Targets and hypothesised modes of action of some antifungal drugs. (Adapted from Rippon and Fromtling, 1993).

The metabolic differences between hosts and their fungal pathogens provide selective targets for chemotherapeutic attacks. The target may be present both in the host and fungal cells but it is inaccessible in the host cells or be totally absent in the host. Subcellular entities that may be injured reversibly or irreversibly are the cell wall, plasma membrane and peroxisomes. Damage to any of these organelles may affect cellular function in general (Kerridge and Vanden Bossche, 1990).

1:6:1 Fungal cell wall as an antifungal target

The generation of new wall material during the growth of a fungal cell is due to a complex balance of biological mechanisms, the alteration of which can lead to fungal cell death. These alterations may be exploited for designing new antifungal drugs which are fungicidal. The cell wall performs vital functions such as preserving the cell, protecting the protoplasm, enabling the fungal cell with the ability to exist in more than one morphological form which is necessary for nutrition, mating, reproduction, dispersal and host invasion (Herrera and Sentandreu, 1989). Because of these vital functions the fungal cell wall is an ideal target for antifungal agents. Another advantage of the fungal cell wall is that antifungals do not have to traverse the plasma membrane to reach their target, and many of the wall components are not found in mammalian cells. Of all cell components, it is the cell wall that comes into contact with the host, and it is this contact of the cell wall with the stratum corneum which is the initial stage in dermatophytosis (Aljabre *et al.*, 1993).

Fungal cell walls contain chitin, glucan and mannan as well as a group of associated proteins (Herrera and Sentandreu, 1989). A number of potential targets in the fungal cell wall for antifungal drugs have been identified that are absent from host cells (Hector, 1993). The enzymes controlling cell wall dynamics provide some of the best targets for antifungal drugs. For example the enzymes involved in the synthesis and hydrolysis of chitin (a polysaccharide

absent in mammals). Enzyme β–1,3–glucan synthetase responsible for the synthesis of cell components present in pathways of intermediary metabolism (Kerridge and Vanden Bossche, 1990).

Since chitin is absent from mammalian cells, chitin synthetase is an ideal target for antifungal drugs. Two chitin synthetases have been identified in membrane preparations obtained from *Saccharomyces cerevisiae*. The structural genes for chitin synthetases I and II namely *CHS1* and *CHS2* have been cloned and sequenced (reviewed in Nombela *et al.*, 1992). The availability of the genes should lead to the identification of targets based on inhibition of chitin synthesis. Two chitin synthetase inhibitors, the polyoxins and nikkomycins are under investigation. Nikkomycin is not effective against *Candida* species but has activity against other species of lesser clinical importance (Hector, 1993).

Enzyme β–1,3–glucan synthase is responsible for the synthesis of cell wall β–glucans. This enzyme is inhibited by compounds with complex chemical structures such as papulacandin, echinocandin and aculeacin (Hammond, 1993). Except for cilofungin, none of these compounds or their derivatives have so far been developed as a clinical drug. Cilofungin, is a modified lipopeptide derived from echinocandin with a restricted antifungal activity and is fungicidal for a variety of *Candida* species. The molecular target for cilofungin is the enzyme β–1,3–glucan synthase of the fungal cell wall. It is being evaluated as a treatment for fungal infections in humans (Hector, 1993). Inhibitors of chitin synthase and glucan synthase have been effective against medically important fungi but have not been used clinically due to various problems such as toxicity, low solubility and limited penetration to the fungal target.

Enzymes involved in the biosynthesis of polysaccharide constituents of the fungal cell wall are ideal targets for therapeutic attack, but no antifungal drug which specifically inhibits polysaccharide synthesis is in clinical use. Membrane associated proteins carry functions in fungi which do not occur in

mammals and hence are ideal as potential targets for antifungal drugs (Kerridge and Vanden Bossche, 1990). Many of the constituent macromolecules of the plasma membrane act as specific targets for antifungal drugs and play a role in the uptake of intracellular target drugs (Kerridge and Vanden Bossche, 1990).

1:6:2 Ergosterol biosynthesis inhibitors

Ergosterol is the main sterol in fungal cells and plays a major role architecturally and functionally in being a prerequisite for cell proliferation. Therefore, the ergosterol pathway has been recognised as a major target for antifungal drugs. Perturbation of ergosterol biosynthesis has a drastic effect on fungal cell metabolism and leads to cell growth inhibition or even cell death. Except griseofulvin, most of the presently available antifungals are claimed to interfere with enzyme systems involved in the synthesis of ergosterol and hence affect the integrity of the plasma membrane indirectly (Polak, 1990). Ergosterol biosynthesis inhibiting (EBI) antifungals constitute one of the most important groups of compounds developed for the treatment of fungal diseases. Three classes of ergosterol biosynthesis inhibitory antifungals are currently in use. They inhibit the ergosterol biosynthesis pathway situated in the endoplasmic reticulum at different points.

1:6:2:1 14α-demethylase inhibitors

Azole antifungals, imidazole derivatives (topical miconazole, clotrimazole, econazole and oral ketoconazole) and the triazole derivatives (itraconazole, fluconazole, saperconazole), both belong to the class of 14α-demethylase inhibitors. They interfere with the cytochrome P-450-dependent 14α demethylation of lanosterol, a key step in the biosynthesis of ergosterol, leading to ergosterol depletion and the concomitant accumulation of 14αmethyl

sterols in the fungal cytoplasmic membrane (Vanden Bossche, 1985). The accumulation of C_{14} methylated sterols in the cell induces permeability changes in cell membrane bound enzymes, inhibition of growth and cell death. The cytochrome P-450 isoenzyme $P\text{-}450_{140Dm}$ is considered the primary target for azole antifungal agents (Yoshida, 1988). The azoles bind by their basic nitrogen to the heme iron and by their N-1 substituent to the protein moiety of fungal P-450 and compete in this way with oxygen binding and activation. This results in the inhibition of P-450 catalysed reactions (Yoshida, 1988).

Most of the available imidazoles, such as, miconazole, clotrimazole, and econazole are used topically. Ketoconazole is the first orally active azole derivative to have been developed and is active against yeasts, dermatophytes and dimorphic fungi (Vanden Bossche, 1985). It, however, effects human androgen biosynthesis at high doses and may cause symptomatic hepatitis (Vanden Bossche, 1985). Itraconazole is a triazole which is effective against a range of superficial mycoses including dermatophyte infections, superficial candidosis and pityriasis versicolor. It does not show any significant metabolic interaction with mammalian cytochrome P-450 dependent enzymes and the absence of drug interactions which are seen with ketoconazole. Itraconazole is also effective against some of the major endemic mycoses. Fluconazole is another triazole effective in superficial and some systemic mycoses (Hay, 1991).

Recent developmental work in this group of drugs has concentrated on enhancing activity, either by speed of action or spectrum of activity and reducing the risk of human toxicity. Extensive modifications of the N-1 substituents and screening of new compounds is being undertaken (Yoshida, 1988).

1:6:2:2 Squalene epoxidase Inhibitors

The allylamines (terbinafine and naftifine) represent a structurally and functionally new and distinct chemical class of synthetic antifungal agents with a broad spectrum of action *in vitro* and primary fungicidal action against the majority of pathogenic fungi. They possess a novel mechanism of action. The allylamines inhibit ergosterol biosynthesis with the accumulation of squalene thus indicating the enzyme squalene epoxidase as the primary target of these drugs (Ryder, 1991). Although ergosterol synthesis is only partially (approximately 20%) inhibited, but cell growth is completely arrested, suggesting that the fungicidal effect of allylamines is related to the heavy accumulation of squalene in treated fungi which at high concentrations is toxic to the fungus (Ryder and Mieth, 1992). Detailed studies of the correlations between cell death, growth inhibition and lipid content in several fungi treated with allylamines have confirmed that the fungicidal action is associated with squalene accumulation in both *C. parapsilosis* and *T. mentagrophytes.* When *Trichophyton* is treated with terbinafine at its MIC, cell death coincides with a massive rise in intracellular squalene concentration, while ergosterol is only slightly depleted (Ryder and Mieth, 1992).

The exact mechanism of toxicity of squalene in fungal cells is not clear. It is thought to increase membrane fluidity leading to the disruption of enzyme function and cell structure or disruption of the fungal cell membrane (Ryder and Mieth, 1992). Ultrastructurally terbinafine has been shown to induce pores in arthroconidia and germ tubes of *T. interdigitale* leading to their destruction (Rashid *et al.*, 1993). Fungi treated with naftifine or terbinafine reveal the presence of large numbers of lipid bodies in the cytoplasm and also in the cell wall probably defining the location of accumulated squalene (Rashid *et al.*, 1993).

Terbinafine is the most potent antifungal allylamine to date. It possesses the characteristics of naftifine but is far superior. The antifungal spectrum of

activity *in vitro* affects a large number of medically important fungi of different genera and species, including dermatophytes, moulds, dimorphic fungi and yeasts. Terbinafine has a uniform high activity against dermatophytes indicating a high specificity which is underlined by the primary fungicidal activity against these organisms. There is a clear correlation between the inhibition of squalene epoxidase and inhibition of growth. Although the squalene epoxidase of *C. albicans* and *C. parapsilosis* are almost equisensitive to terbinafine, it is fungicidal against *C. parapsilosis* and fungistatic against *C. albicans* (Shear *et al.*, 1992).

Ryder (1991) has suggested that fungal species differ in their sensitivity due to a decreased availability of ergosterol and also in their reaction to the intracellular accumulations of squalene. Terbinafine concentrations required for growth inhibition of the yeast form of *C. albicans* are 100 fold greater than those effective against dermatophytes. Even at very high concentrations of the allylamines, squalene accumulation in *C. albicans* is only a fraction of that observed in dermatophytes. The incomplete inhibition of cell growth which reaches only about 80% parallels the reduction in ergosterol content. Thus, ergosterol deficiency appears to be the mechanism of action causing the fungistatic effect by terbinafine against *C. albicans* (Ryder and Mieth, 1992). Squalene epoxidation and lanosterol demethylation are important steps not only in the biosynthesis of ergosterol in fungi but also in the formation of cholesterol in mammals (Poralla, 1982). Studies of the effects of terbinafine on squalene epoxidase from mammalian and fungal cells show that terbinafine is three to four orders of magnitude more selective for the fungal enzyme and that the drug is a specific potent non-competitive inhibitor of this enzyme avoiding toxicity to the squalene epoxidase enzyme system in mammalian cells (Ryder and Mieth, 1992). This greatly enhances the usefulness of the compound as a systemically acting drug. The high selectivity for a single fungal enzyme and the apparent lack of interference with other enzymes of fungal and mammalian origin uniquely distinguish allylamines from the other main group of ergosterol

biosynthesis inhibitors, the azoles. Cross resistance to allylamines rarely develops as they have a completely different mode of action (Ryder and Mieth, 1992).

Fungicidal action is an important attribute of an antimycotic, contributing to rapid therapeutic action and is of particular significance for the treatment of immunocompromised patients. Experimental evidence indicates that the allylamines have the potential to exert fungicidal action during therapeutic application. Along with this novel mechanism of antifungal action all of these properties ensure that further work on this class of compounds holds out interesting prospects for the development of novel and highly active antifungal agents.

There is a miscellaneous group of antifungals which includes a number of useful drugs. The thiocarbamate antifungals, tolnaftate and tolciclate exhibit strong antifungal activity against dermatophytes only. They inhibit ergosterol biosynthesis leading to an accumulation of squalene, the primary target being squalene epoxidase (Polak, 1990).

Amorolfine, is the first and only morpholine derivative in use as a topical antifungal. It possesses a broad spectrum of activity which includes dermatophytes, yeasts, dimorphic fungi and moulds and is not only fungistatic but fungicidal against most species (Polak, 1990). Amorolfine interferes with ergosterol biosynthesis at two steps, the Δ_{14} reduction and the Δ_{7-8} isomerisation. As a consequence of this inhibition the Δ_{14} sterol ignosterol is accumulated in the cell membrane and ergosterol is depleted (Polak, 1990). In experimental models of systemic mycoses, amorolfine shows no significant activity. It has a long retention time in the horny layer of the skin and shows high efficacy as a topical treatment in fungal skin infections especially onychomycosis.

All the ergosterol biosynthesis inhibitors prevent fungal growth and induce modification of the cell morphology. It is the depletion of ergosterol

rather than the structure or amount of unusual membrane components which adversely affects the cells.

1:7 Identification and evaluation of novel targets for antifungal drugs

The difficulties which have been experienced in identifying specific fungal targets are due to the functional conservation of protein sequence which encompasses all eukaryotic organisms. In relatively conserved systems the differences between fungal and mammalian sequences are sufficient to permit selective attack on essential fungal functions (Rippon and Fromtling, 1993).

A target for anti-*Candida* drugs is the peptide transport system in *C. albicans.* The peptide transport system facilitates the movement of molecules into *C. albicans.* However, there has been a very limited approach in using this system to design drugs as the carrier must be stable in the host and the carrier or carrier–toxic agent linkage must be degradable inside the target cell (Naider and Becker, 1988). It has not been possible to design "the optimal peptide carrier". Polyoxins and nikkomycin, which are chitin synthetase inhibitors, enter *C. albicans* via a peptide transport system but they do so with very different efficiencies. The use of chitin synthase inhibitors with a greater affinity for the peptide transport system is a target to be further investigated for new antifungal drugs since mammalian cells do not contain any enzymes that are analogous to chitin synthetase of *C. albicans* and that this protein appears to be an ideal target for a cell specific inhibitor. Further developments in this area depend on the appropriate choice of targets, the ability to overcome strain variations, differential target cell and host peptidase activity (Naider and Becker, 1988). Active transport systems provide an interesting and attractive alternative in the rational design of such drugs.

Another target is the transport into fungal cells of a drug that is metabolised to an active compound by an enzyme that is absent or has low

activity in the host thus avoiding toxicity to host cells. For example, 5-fluorocytosine is taken up by a cytosine permease and once inside the fungal cell is rapidly deaminated to 5-fluorouracil, a cytotoxic agent. This selectivity occurs because mammalian cells lack the cytosine deaminase responsible for the conversion of 5-fluorocytosine into 5-fluorouracil (Polak, 1990).

The paradimicins, are calcium dependent agents that kill fungal cells without lysing them. They are sterol-like molecules with amino acid containing side chains probably from calcium linked complexes with mannose containing components of fungal cell membrane. This unusual mode of activity renders these agents "relatively non-toxic and broad acting". Moreover they do not cross react or interfere with other antifungal agents. The compounds studied so far have shown no oral availability.

Protein kinase has been implicated in yeast budding and morphogenesis. Since the protein kinase gene has been cloned it has been shown that vital functions crucial and specific for cell wall formation are coded by genes, which are represented by protein kinase genes (Nombela *et al.*, 1992). Disruption of these genes would lead to fungal cell death and would provide knowledge of targets crucial for fungal cell viability which can be used to design new fungicidal antifungal drugs.

Other components of the cell which are being investigated to identify new antifungal targets are the tubulins: a range of tubulin binding agents are currently in use as selective drugs. The fungal tubulins are sensitive to such agents, though the use of these compounds in therapy may be limited by a high degree of fungal resistance to them. Fungal secreted proteinases especially of *C. albicans* have been implicated as virulence factors and are involved in the adherence, tissue penetration, invasiveness and evasion of the immune system (Odds, 1991). Currently, there is no non-toxic inhibitor which can be tested in animal models so as to be developed as a proteinase inhibitor type antifungal drug. Surface components of fungal cells have been suggested as potential targets, possibly a receptor which upon activation can cause cell death. Protein

synthesis can be affected by intervention in translation directed against elongation factor EF3 (reviewed in Koltin, 1989).

Other entities which have potential antifungal activity include cytokines, colony stimulating factor and tumour necrosis factor. There is progress being made in the study of existing antifungal drugs and the development of new compounds within families of existing drugs. Proven antifungal activity of these compounds could be a source of new antifungal therapy.

The technique of gene disruption or gene transplacement developed for non-pathogenic yeasts and filamentous fungi is also being employed for pathogenic fungi. Recent research has shown that multiple genes may be involved in the formation of a functional wall structure in *C. albicans* and a block in the function of these genes is lethal for growing cells (Nombela *et al.*, 1992). Knowledge of these "lethal events" which lead to cell death will be most useful in identifying novel targets for antifungal drugs. In future the genes which encode these functions could be considered as targets for antifungal therapy.

1:8 Aim of the study

Dermatophytes tend to grow in the keratinized tissues of the body, stratum corneum, nail and hair. Little is known about the events involved in the colonization and invasion of these tissues by dermatophytes, and how antifungal drugs interact with these fungi in keratinized tissues. This study was undertaken to determine the early events involved in the invasion of keratinized tissues by dermatophyte fungi and also to observe the dermatophyte fungi-antifungal interaction in these tissues and explore the possibility of developing novel *in vitro* models for assessing antifungal drugs, which simulate the physiological environment of dermatophyte fungi.

The importance of arthroconidia as the main infectious propagules of dermatophytes and the major mediator of disease transmission in dermatophytes has been clearly established (Chapter 1, sections 1:3:1 and

1:3:2). As arthroconidia are most likely to play a major role in the persistence and spread of dermatophytosis they are the most relevant morphological form of dermatophytes to be used in experimental studies of dermatophyte infection. Therefore pure arthroconidia were prepared and used in this study.

The aims of this study were:

1. To study the germination and hyphal extension of dermatophyte arthroconidia on stratum corneum from different body surfaces and determine the sequence of events involved in the formation of mycelium at these sites. This work would help in understanding the process of skin invasion by dermatophyte fungi.
2. To investigate the effect of the antifungal drug terbinafine on the germination and hyphal extension of arthroconidia on stratum corneum and observing morphological changes in the growth forms, arthroconidia and germ tubes following exposure to terbinafine. This work would help in clarifying how antifungal drugs work against dermatophyte fungi in tissues. Also exploring the possibility of using this stratum corneum model for testing antifungal drugs against dermatophytes.
3. To study the morphological transformation of arthroconidia on nails and the process of nail invasion by dermatophyte fungi. This would help in understanding more about the growth of dermatophytes on keratinous materials.
4. To investigate the nail model for studying the activity of antifungal drugs against dermatophytes. This would help to elucidate how antifungal drugs penetrate the nail plate and their mode of action in tinea ungium.
5. To utilise a novel *in vitro* model for studying the process of hair invasion by dermatophyte fungi and the mode of action of antifungal drugs in tinea capitis. This would help in understanding more about how dermatophytes colonize and invade keratinized tissues and the dermatophyte–antifungal drug interaction in these tissues.

6. To explore the possibility of developing a human skin equivalent model into a system for assaying the efficacy of antifungal drugs.

This study would provide a detailed insight into the kinetics of skin, nail and hair invasion by dermatophyte fungi and help in understanding the biology of dermatophytes and pathogenesis of dermatophytosis. At the same time clarifying and improving our knowledge of how the dermatophyte fungi–antifungal drug interaction takes place in the tissues. The possibility of developing new, simple, rapid and inexpensive models for further assessment of antifungal drugs on a wider scale is proposed.

Chapter 2

Invasion of the stratum corneum by dermatophyte fungi

2:1 Summary

Germination of dermatophyte arthroconidia and hyphal extension on stratum corneum from different body surfaces was studied. Arthroconidia of one strain of *T. interdigitale* and stratum corneum from face, back of hand, palm, leg and sole were used. Four hours after inoculation, arthroconidia had begun to germinate. Germination was initially slow and gradually increased with time to reach 100% by 24h of incubation on face and back of hand and in the range of 80–95% on palm, leg and sole. The germ tubes extended across the stratum corneum with side branches appearing by 16h. There was a direct relationship between percent germination and germ tube length on face, back of hand and leg and an inverse relationship on palm and soles. By 48h vegetative mycelia were observed on all body sites examined. Scanning electron microscopy revealed germinated and ungerminated arthroconidia adhering to corneocytes. The germ tubes were lying on the stratum corneum in close apposition to the corneocytes, in areas penetrating them and forming tunnels in the stratum corneum. Transmission electron microscopy revealed close apposition between arthroconidia and corneocytes with a fibrillar–floccular material between them. Factors released by corneocytes appear to be crucial in the establishment of dermatophyte infection.

2:2 Introduction

Germination of arthroconidia is an important and essential stage in the establishment of dermatophytosis (Aljabre *et al.*, 1992a; Richardson and Aljabre, 1993). Arthroconidia after adhering to corneocyte of the stratum corneum and failing to germinate are shed from the surface by the skins exfoliative process and lesions are not produced (Richardson and Aljabre, 1993). The growth of emerging germ tubes is apical and for an active dermatophyte infection to occur germ tubes must be able to penetrate the

stratum corneum (Aljabre *et al.*, 1992a) and grow peripherally to establish a mycelium.

Study of the interrelationship between the stratum corneum and the growth of dermatophyte is necessary for a complete understanding of the process of dermatophytosis. There have been many attempts to produce dermatophyte infections *in vivo* and *in vitro*, (Strauss and Kligman, 1957; Singh, 1973; Aljabre et al., 1992a). Experimental human infections have been carried out with various degrees of success (reviewed in Knight, 1972) and varied forms of inoculum have been used including mycelial suspension, "spore suspensions" and hyphal fragments. The skin has been occluded and macerated to encourage the establishment of infection, (Singh, 1973). Similarly, animal models have been used to produce dermatophyte lesions (Chittasobhon and Smith, 1979; Fujita and Matsuyama, 1987).

In vitro experimental models for the study of dermatophyte infection are few and consist of stripped layers of stratum corneum. Marks and Dawber (1971) introduced the skin surface biopsy technique consisting of stripping the stratum corneum by cyanoacrylate adhesive to study the biology of infection of the stratum corneum. Whiting and Bisset (1974) found this technique to be simple, quick and reliable in clinical diagnosis of superficial fungal infections. Knight (1973a) used sheets of stratum corneum stripped by an adhesive tape to study the growth of dermatophyte microconidia and to observe the effect of temperature and humidity on the growth of *T. mentagrophytes* spores (Knight, 1976). Aljabre *et al.* (1992a) studied the growth of arthroconidia on stripped sheets of stratum corneum. As arthroconidia are the main infective propagules of dermatophytes and the only form of parasitic conidia found in tissues (Hashimoto, 1991) they are the most relevant conidia to be used in experimental studies of dermatophyte infections. However, very few studies (Aljabre *et al.*, 1992a) have studied their germination on body surfaces and the relationship between germination and hyphal extension on different body surfaces. The exact sequence of events in the formation of a vegetative

mycelium on different body surfaces has not been studied before, neither has this process been studied ultrastructurally in detail. Rashid *et al.* (1993) used D–SQUAMES for stripping layers of stratum corneum and found this model to be superior for the ultrastructural study of dermatophytes. D–SQUAMES are a simple, rapid and inexpensive method of studying the biology of dermatophyte infection.

Stratum corneum is the target tissue of the dermatophytes and the use of stripped sheets of stratum corneum to study dermatophyte infection, is appropriate where the structural integrity of the tissue is maintained under a number of environmental conditions which bear a close resemblance to the *in vivo* situation.

2:3 Aim of the study

The aim of the study was to investigate the ability of dermatophyte arthroconidia to germinate on different body surfaces and to follow their growth and the formation of a vegetative mycelium on these sites. Also to study the ultrastructural relationship between arthroconidia and germ tubes while growing on the stratum corneum. This work would help in understanding the kinetics of the invasion of stratum corneum by dermatophyte fungi while the structural integrity of this tissue is maintained.

2:4 Materials and methods

2:4:1 Dermatophyte strain

A clinical isolate of *T. interdigitale* (Lab No. D5777OA) was selected on its ability to produce pure arthroconidial cultures. The strain of *T. interdigitale* was a fresh isolate from a clinical specimen (skin scales) sent to the Regional

Mycology Reference Laboratory, Department of Dermatology, University of Glasgow, U.K.

2:4:2 Cultivation and preservation of culture

The growth medium used for the subcultures and maintenance of stock cultures was glucose peptone agar (GPA) (Appendix). The strain of *T. interdigitale* was recovered on GPA at 28°C. Subcultures were prepared on GPA and incubated at 28°C for 10 days to obtain an optimal yield of microconidia.

2:4:3 Production of arthroconidia

Ten day old colonies of *T. interdigitale* grown on GPA were scraped off with a sterile surgical blade and dispersed into sterile distilled water. The suspension was mixed vigorously for three minutes to disperse clumps. One ml of this suspension was plated evenly on GPA plates with a sterile glass rod and the plates placed in a modular incubator chamber (Flow Laboratories Fig 2:1) in an atmosphere of 10% CO_2, 90% O_2 and incubated at 37°C (Emyanitoff and Hashimoto, 1979). The incubation module was flushed through daily with 10% CO_2, 90% O_2. Incubation was carried out for 10 days by which time abundant arthroconidial formation had occurred and the colonies were composed of disarticulated arthroconidia (Fig 2:2).

2:4:4 Preparation of arthroconidia

Arthroconidia were prepared as a pure single cell suspension from 10 day-old cultures grown as surface lawns on GPA in an atmosphere of 10% CO_2, 90% O_2 at 37°C. Surface growth was harvested from the culture plate with a sterile scalpel blade, suspended in 5 ml 1% (v/v)Tween 80-distilled

water (Appendix) and agitated on a vortex mixer for 5 min. The arthroconidia were washed twice in Tween 80–distilled water and then filtered through column chromatography grade glass wool to remove chains and aggregates of arthroconidia. The arthroconidia were then adjusted to a concentration of $5x10^4$ cells/ml with distilled water. This procedure resulted in a pure, uniform suspension of single cells (pH 6.5). No damage to the arthroconidia had occurred. Viability of singlet arthroconidia was determined by inoculation on stratum corneum fixed to D–SQUAME discs for 24h at 28°C. An arthroconidium was considered to have germinated when a visible germ tube had developed. Ten arthroconidia selected at random were assessed. The percentage of germinated arthroconidia was determined in triplicate per experiment.

2:4:5 Preparation of stratum corneum sheets

Sheets of stratum corneum were removed onto D–SQUAME skin surface sampling discs (CuDerm Corporation, Dallas, Texas, USA) by repeated application of the disc to the skin until they became non–adhesive. The discs were then mounted on glass slides with the adhesive side uppermost. Stratum corneum was obtained from a volunteer from the following body areas: face, back of hand, palm, leg and sole. It was ascertained that no previous antifungal medication had been administered. No prior antiseptic swabbing of the body areas was done.

2:4:6 Germination of arthroconidia on stripped sheets of stratum corneum from different body surfaces

Sheets of stratum corneum from face, back of hand, palm, leg and sole were prepared and inoculated with 50μl of a $5x10^4$ arthroconidia per ml suspension. D–SQUAMES were placed on glass slides supported on bent

glass rods in a sterile plastic petri dish containing filter paper soaked in 2 ml distilled water and incubated at 28°C for varying lengths of time: 4, 16, 20, 24h.

D-SQUAMES were covered with cover glasses and examined microscopically under x400. Control arthroconidia were inoculated onto D-SQUAMES without stratum corneum and incubated at 28°C for various time intervals. Percent germination was determined by counting the number of arthroconidia with a visible germ tube in a field of view out of ten arthroconidia counted. The counting was done in triplicate in three separate cultures and the values expressed as the average mean ± SD.

2:4:7 Hyphal extension on stripped sheets of stratum corneum from different body surfaces

Sheets of stratum corneum prepared and inoculated as in section 2:4:6 and incubated at 28°C for varying lengths of time: 16, 20, 24, 48h, 5 days and 7 days. For control purposes arthroconidia were inoculated onto D-SQUAMES without stratum corneum and incubated at 28°C for various time intervals. Germ tube length was measured by a micrometer eye piece graticule. 3-4 germ tubes were counted at random at each reading. One division on the eyepiece = 2.5μm.

2:4:8 Effect of temperature and humidity on the germination of arthroconidia

Sheets of stratum corneum from the back of the hand were prepared and point inoculated with 50μl of a $5x10^4$ arthroconidia per ml suspension of *T. interdigitale*. Strips were incubated for 24h under the following conditions: 4°C, 45°C and in the absence of moisture. Control D-SQUAMES were prepared without stratum corneum and point inoculated with 50μl of the above suspension, were also incubated under the above conditions.

2:4:9 Electron microscopy of arthroconidia and germ tubes growing on stratum corneum

Strips of stratum corneum from different body sites removed onto D-SQUAMES and inoculated with ungerminated arthroconidia of *T. interdigitale* were incubated at 28°C for various time intervals as has been described in section 2:4:6 and then fixed in 3% (v/v) glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4 for 1h. After washing in 0.1M sodium cacodylate buffer for 1h the adhesive discs were post fixed in 1% (w/v) osmium tetroxide and rinsed again in buffer followed by successive dehydration steps in graded ethanol solutions (25–100%) substituted with propylene oxide (2 x 10min) and then embedded in araldite (Glauert, 1975). Sections, 2μm in thickness, were cut and stained with toluidine blue for light microscopy. Ultrathin sections, 700nm in thickness, were cut from selected areas, mounted on copper grids, stained with uranyl acetate and lead citrate. Specimens were examined in a Jeol 1200Ex transmission electron microscope.

For scanning electron microscopy specimens were fixed in glutaraldehyde and dehydrated in graded ethanol solutions as above, substituted with liquid carbon dioxide, dried in a critical point dryer, mounted on 10mm aluminium stubs, sputter coated with gold and examined in an JSM 6400 scanning electron microscope.

2:5 Results

2:5:1 Growth of arthroconidia and germ tubes on stratum corneum from different body surfaces

The percent viability of arthroconidia calculated from triplicate assays of *T. interdigitale* was 100±0. First evidence of germination of arthroconidia appeared around 4h incubation at 28°C. Germination was initially slow and

increased gradually upon further incubation up to 24h by which time nearly 100% arthroconidia had germinated on the face, back of hand and 95% on the leg. On the palms and soles, arthroconidial germination was very slow at the start, variable and unpredictable, and reached between 80–90% by 24h (Fig 2:3). No evidence of germination occurred on any of the body sites examined before 4h incubation at 28°C. At 16h, % germination was high on face and leg compared to other sites (Fig 2:3). Maximal arthroconidial germination and germ tube growth was observed on the face, followed by back of the hand at 24h (Fig 2:3–4). Adherent and non–adherent arthroconidia had germinated and some arthroconidia showed multiple germination. Germ tubes emerged from any part of the arthroconidial surface. Prior to germination an increase in size of arthroconidia was observed. Exact quantification of percent germination was difficult before 16h as the arthroconidia were few and germ tubes were short. At 24h it was not possible to quantify germination because of the formation of micro–colonies.

Figure 2:4 shows the germ tube length on different body sites in relation to incubation time. Between 16h and 24h germ tube length increased by almost twice. Compared to 16h and 20h apparent differences were seen in the germ tube lengths on all sites (face, back of hand, leg and palm and soles).

At 16h, germ tubes were small but well formed with side branches starting to appear. By 20h germ tubes were long and further extended with single or multiple side branches arising from different parts of the germ tube and secondary germlings appeared (Fig 2:5). Germ tubes were comparatively shorter and thinner on palms and soles with fewer side branches compared to the other sites examined. By 20h the side branches started to intermingle on face, back of hand and leg forming small microcolonies while on the palm and sole no microcolonies were observed.

By 24h the germ tubes had formed a mycelium on the face, back of hand and leg which made further quantification of germ tube length impossible. By 40h the vegetative mycelium was well established at these sites and the

hyphae extended peripherally, with apical hyphal growth. By 5 days the hyphae had started to show signs of segmentation and arthroconidia formation. On the palm and soles germ tube extension was variable and tended to decrease after 20h (Fig 2:4) and the formation of a mycelium was unpredictable. Occasionally a mycelium was noticed on these sites around 48h.

Germination and germ tube length growth correlated strongly on face, back of hand and leg, where the % germination increased from 16h onwards so did the germ tube length at these sites. On the palms and soles although percentage germination was increasing from 16h onwards, there was a fall in germ tube length after 20h (Fig 2:4). Control plates (that is, arthroconidia inoculated onto D-SQUAME without stratum corneum) showed poor arthroconidial germination at 24h following incubation. Germ tubes were short, thin with no side branches with a tendency for arthroconidia and germ tubes to clump together with no directional spread of germ tubes. Hence the controls were not quantified further.

2:5:2 Ultrastructure of arthroconidia and germ tubes growing on stratum corneum

Transmission electron microscopy revealed that 5 or more layers of stratum corneum were removed by the D-SQUAME discs. Transmission electron microscopy of arthroconidia incubated for 16h showed oval, spherical or cylindrical forms with regular cytoplasmic membranes surrounded by a smooth bilayer cell wall and a fibrillar appearance of the outer wall (Fig 2:6). On scanning electron microscopy germinated and ungerminated arthroconidia were observed. The arthroconidia had a smooth surface. Some conidia still retained remnants of the hyphal wall attached on their surface (Fig 2:7). The arthroconidia were seen lying in close apposition to and attached to individual corneocytes or lying on their surface and firmly adherent to them (Fig 2:8). Transmission electron microscopy revealed close apposition between the

arthroconidia and corneocytes (Fig 2:9). Between the two cell types a fibrillar–floccular material which was also lining the inside of the cervices could be seen. In the stratum corneum model the corneocytes appeared intact and where adherence was seen the crenated appearance of the corneocyte membrane was absent and a smooth attachment area was evident. Similarly, attachment of germ tubes resulted in the same appearance (Fig 2:10).

Scanning electron microscopy showed that corneocytes were hexagonal in shape and their surface was convoluted. At 16h, arthroconidia were seen lying in pockets formed by corneocytes or lying invaginated in between or behind corneocytes (Fig 2:11). Ungerminated arthroconidia were of various sizes, some small and round while others were much larger and oval in shape. They were roughly 10μm in size. Arthroconidia were seen adhering to corneocytes singly, in pairs or clusters. Arthroconidia appeared to attach to corneocytes at either the polar or equatorial regions of their cell wall and as this attachment process progressed at around 20h the microvilli of the corneocyte appeared to flatten and a depression was formed where arthroconidial attachment had taken place (Fig 2:12). Similarly, attachment of germ tubes resulted in the same appearance (Fig 2:13).

On scanning electron microscopy after 16h incubation germ tubes appeared as cylindrical forms with lateral branches and of variable length (Fig 2:14). The germ tubes had a smooth surface with prominent septa. Side branches were evident emerging along the length of the tube. At 20h, germ tubes were seen lying on the corneocytes in close apposition to them while other germlings were adhering to adjacent corneocytes. The side branches and secondary germlings appeared to have an equal capacity to adhere to the corneocyte (Fig 2:15). No apparent damage to the corneocyte was seen. Germlings were seen to penetrate through and in between corneocytes, similarly side branches were seen to penetrate through and in between corneocytes(Fig 2:16). At 24h, hyphal elements appeared to be embedded in the corneocyte surface and germ tubes were seen forming tunnels through

layers of the stratum corneum, by penetrating through and in between corneocytes, similarly side branches were seen to penetrate through and in between corneocytes (Fig 2:17). Multiple side branches arising from the body and apex of germ tube were seen to be growing in a directional way towards the nearest corneocyte (Fig 2:18). Branches were initiated from subapical, apical regions and also from the septa.

At 16, 20 and 24h a mixture of germinated and ungerminated arthroconidia with germ tubes of varying lengths, single and multiple side branches were seen. By 24h a vegetative mycelium was observed (Fig 2:18a). Germinated arthroconidia and germ tubes were seen lying on bare areas of the D-SQUAME. The individual corneocytes demonstrated irregular surface folds, convolutions and occasionally a more villous appearance (Fig 2:19). Some shrinkage of the specimen was seen giving bare areas in between corneocytes. Scanning electron micrograph of D-SQUAMES without corneocytes at 20h showed germinated and ungerminated arthroconidia tending to clump together with no well formed germlings or side branches (Fig 2:20).

2:5:3 Effect of temperature and humidity on the germination of arthroconidia

Germination of arthroconidia on stratum corneum from back of hand did not occur after incubation for 24h in the absence of moisture or at 4°C or 45°C. Germination at 37°C did occur but was not as rapid as at 28°C. Based on these findings and the review in section 2:6:3 further experiments were conducted at 28°C to avoid drying of the D-SQUAMES.

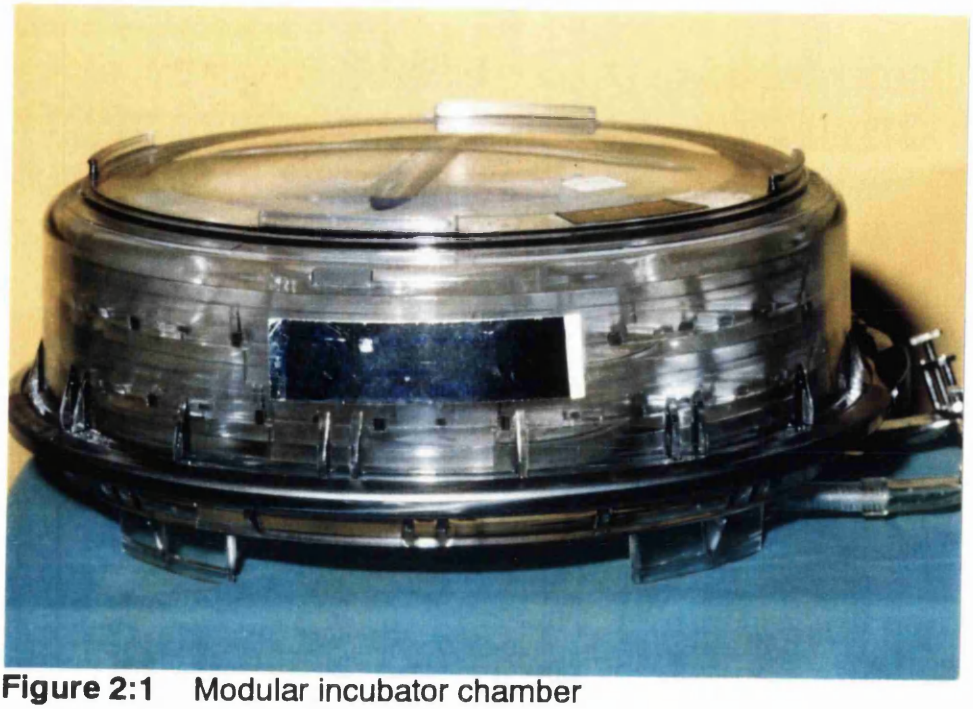


Figure 2:1 Modular incubator chamber

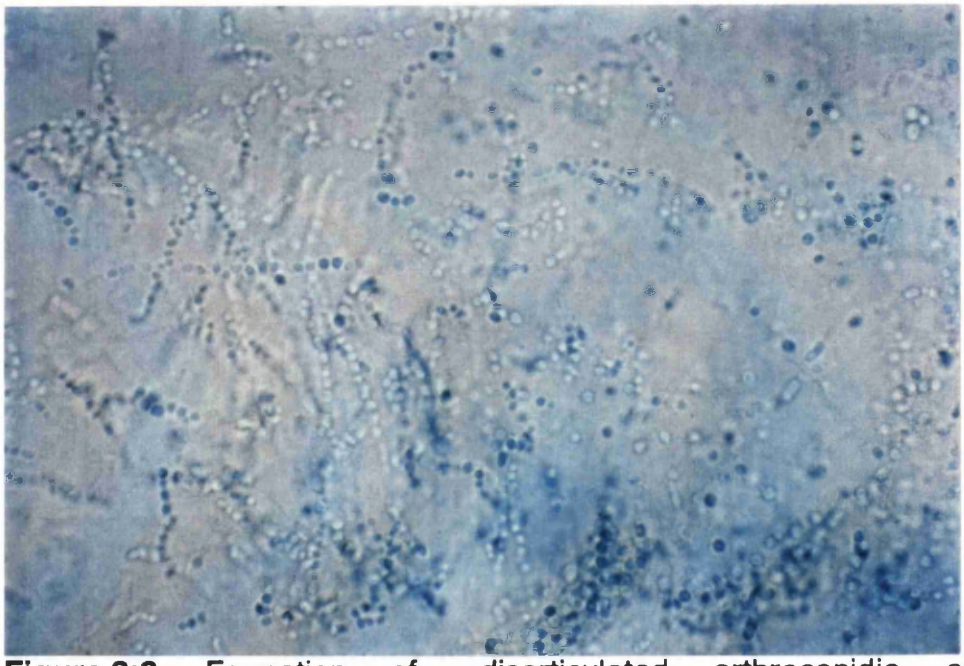


Figure 2:2 Formation of disarticulated arthroconidia of *T. interdigitale* on GPA after 10 days incubation in an atmosphere of 10% CO_2, 90% O_2 at 37°C. Lactophenol cotton – blue mount X250.

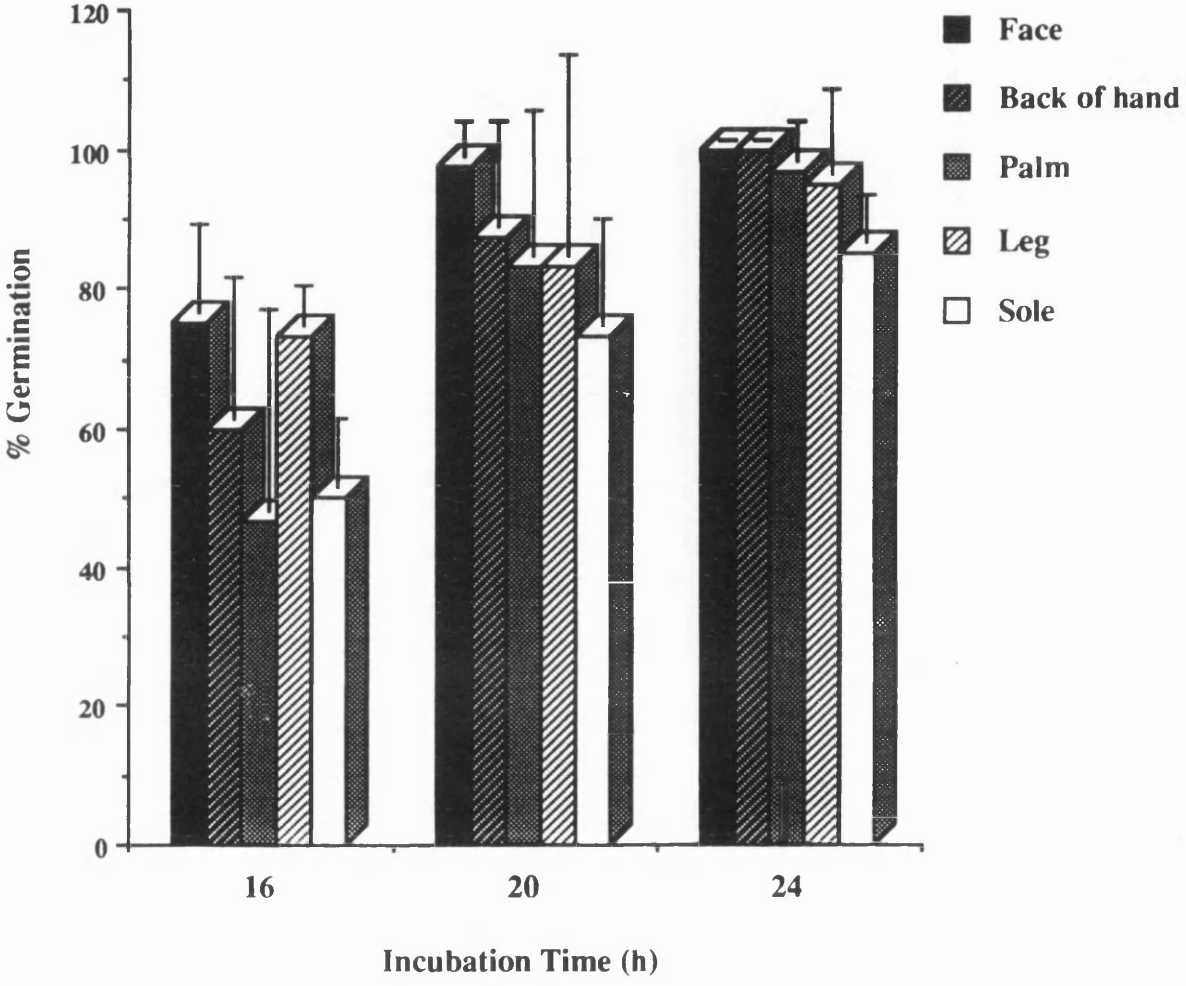


Figure 2:3 % germination of *T. interdigitale* arthroconidia at various time interval on different body sites.
The counting was done in triplicate in three separate cultures and the values expressed as the average mean ± SD –

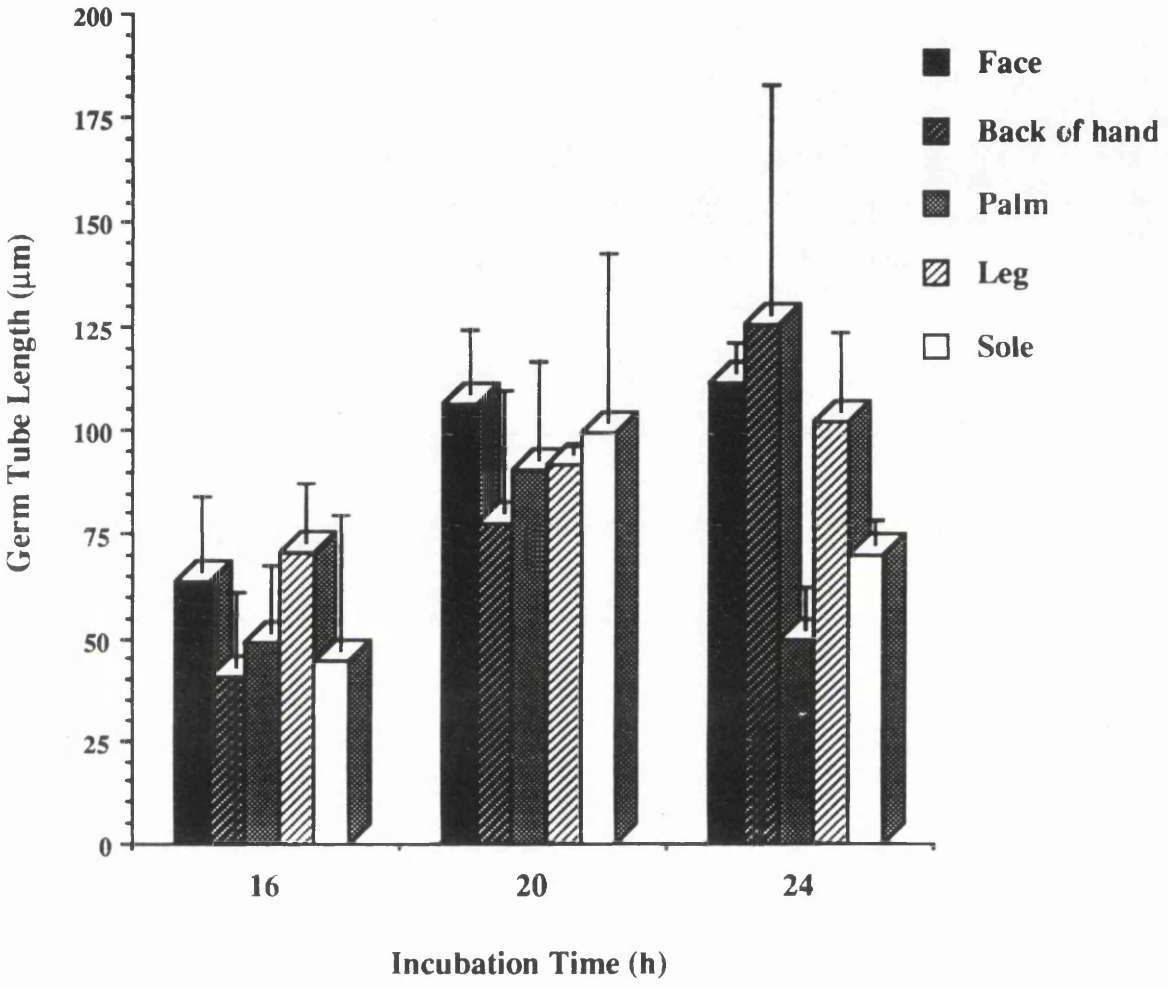


Figure 2:4 Germ tube length of *T. interdigitale* at various time interval on different body sites.
The counting was done in triplicate in three separate cultures and the values expressed as the average mean ± SD

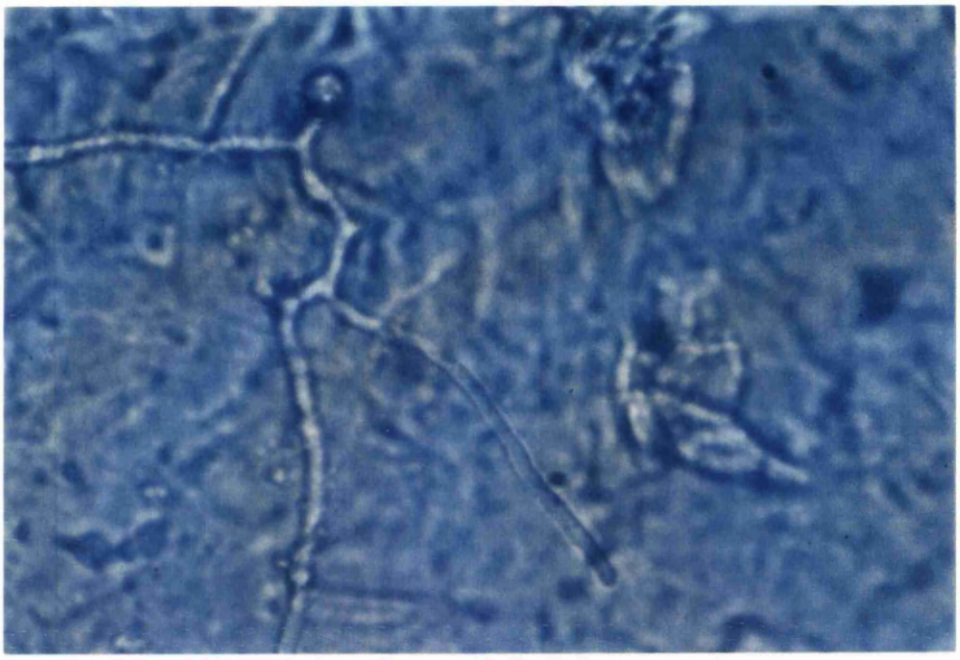


Figure 2:5 Germ tube of *T. interdigitale* after 20h incubation at 28°C. Note the side branches arising along the length of the filament X 400.

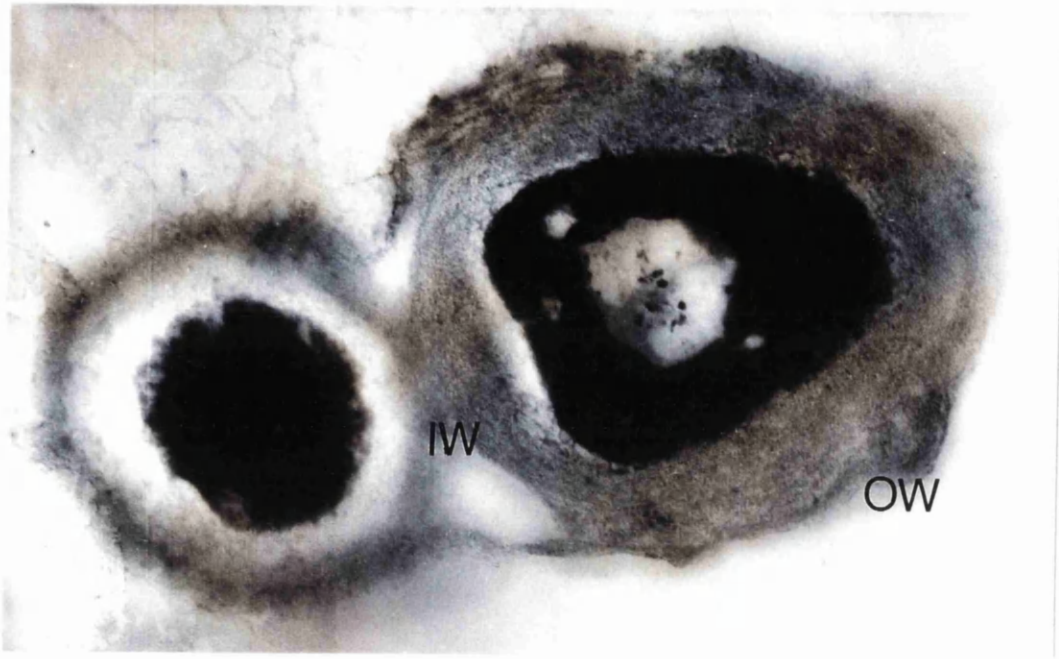


Figure 2:6 Transmission electron micrograph of *T. interdigitale* arthroconidia. The arthroconidia have a thick wall and a well defined plasma membrane. Note the outer wall (OW) is only evident over parts of the cell and gives a fuzzy appearance. The cells are still connected at the inner wall (IW) and have not separated fully X 40000.

Figure 2:7a

Figure 2:7b

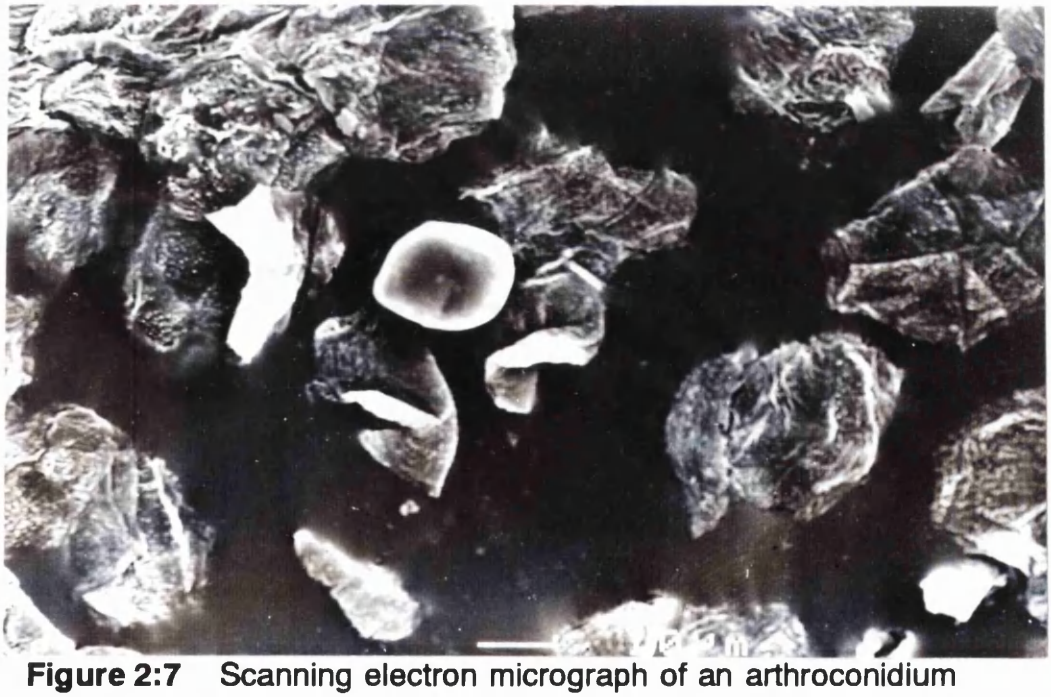


Figure 2:7 Scanning electron micrograph of an arthroconidium of *T. interdigitale* (a) Note the smooth, intact surface of the cell wall and remnants of the outer hyphal wall (arrow) X 4800 (b) Note the adherence to more than one corneocyte X 1700.

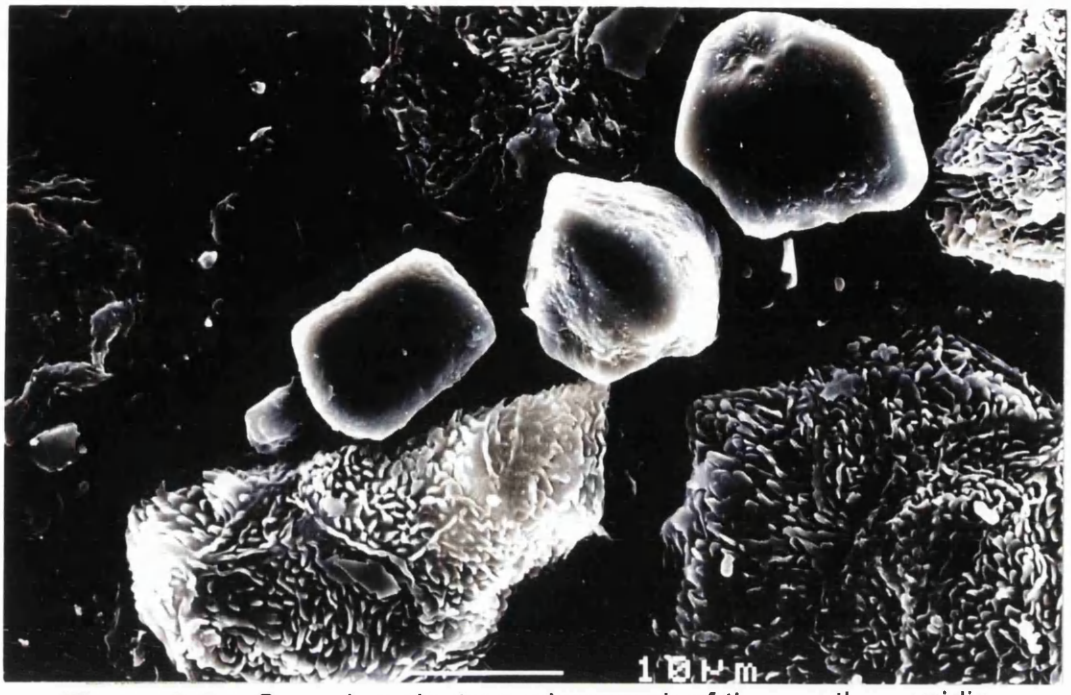


Figure 2:8 Scanning electron micrograph of three arthroconidia of *T. interdigitale* lying in close apposition to corneocytes. Note the convolutions on the corneocytes and adherence to more than one fold of the corneocyte is seen X 3600.

Figure 2:9a

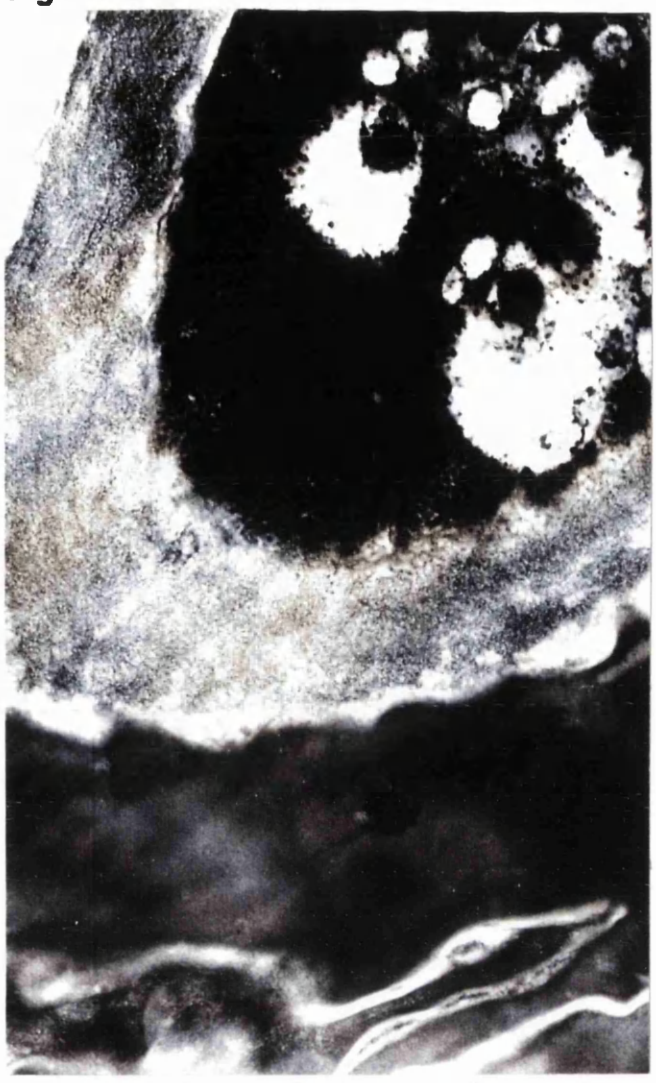
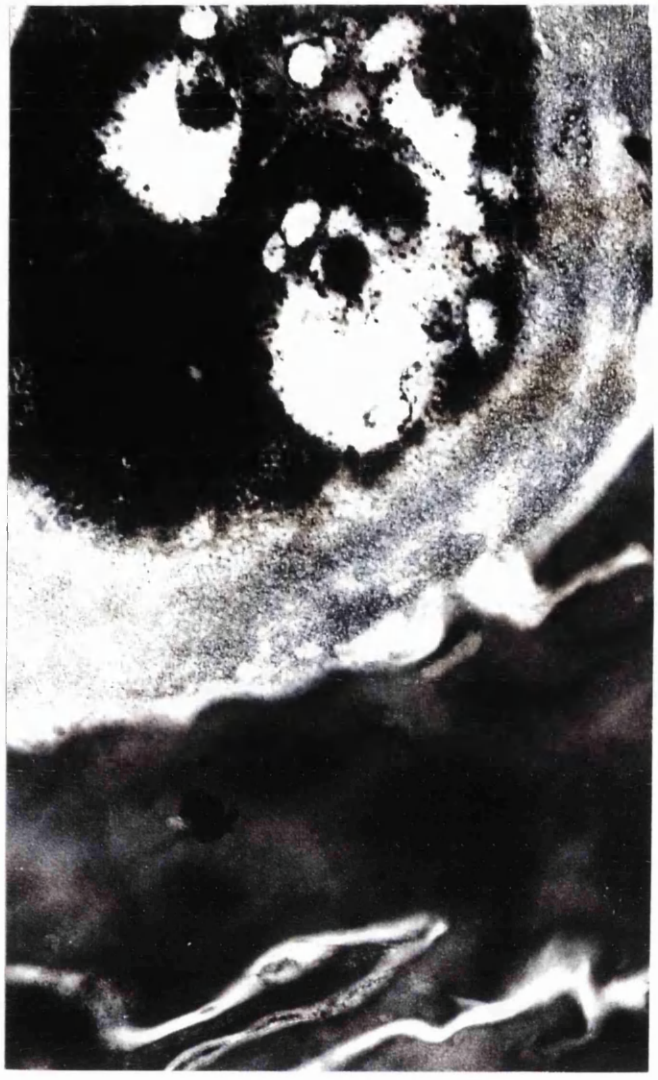


Figure 2:9 Transmission electron micrograph of an arthroconidium of *T. interdigitale* adhering to a corneocyte (a) Note the close apposition between the two cells and the appearance of a fibrillar–floccular material on the arthroconidium surface (b) where adherence is taking place the crenated appearance of the corneocyte membrane is absent and a smooth attachment area is evident. The fibrillar–floccular material is seen to penetrate the cervices in the corneocyte, X 37500.

Figure 2:9b



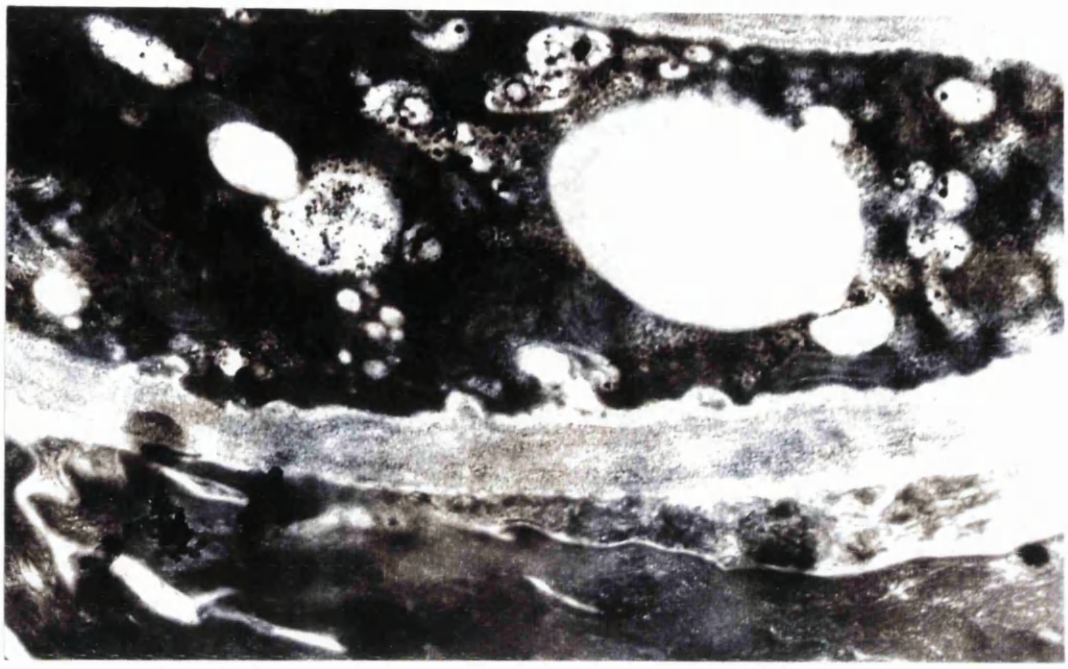


Figure 2:10 Transmission electron micrograph of a *T. interdigitale* germling adhering to a corneocyte. Note the close apposition between the cell wall of the germling and the surface of the corneocyte with flattening of the crenated appearance of the corneocyte membrane X 37500.

Figure 2:11 Scanning electron micrograph showing an arthroconidium of *T. interdigitale* lying in a pocket formed by corneocytes X 3600.

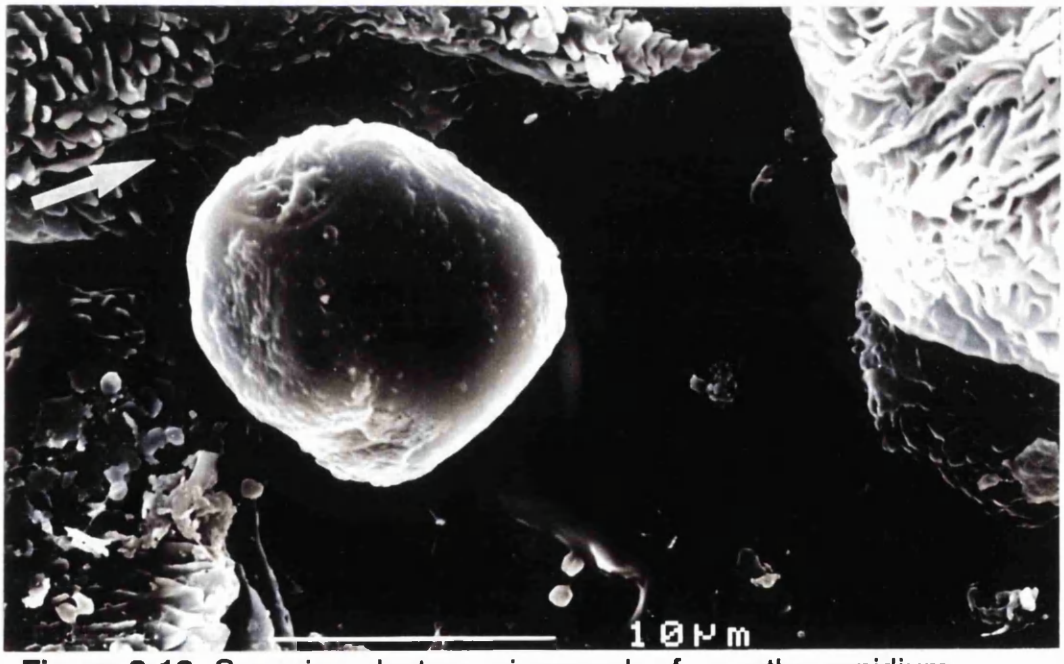


Figure 2:12 Scanning electron micrograph of an arthroconidium of *T. interdigitale*. Note the flattening of the corneocyte microvilli and a depression formed where arthroconidial attachment is taking place (arrow) X 6600.

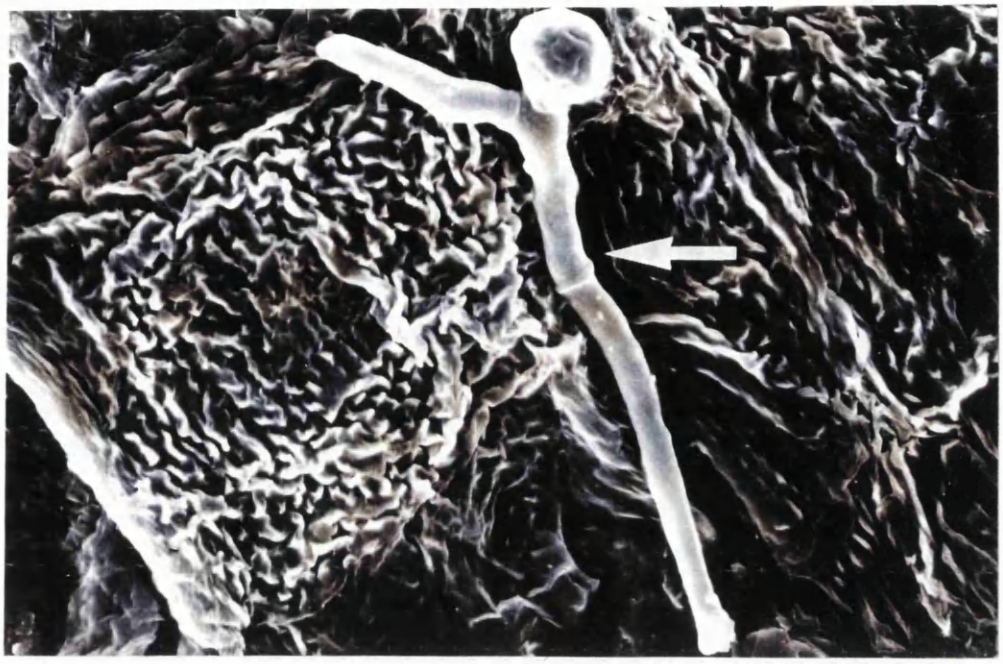


Figure 2:13 Scanning electron micrograph of a germling of *T. interdigitale*. Note the depression formed in the corneocyte where the filament is lying (arrow) X 7400.

Figure 2:14a

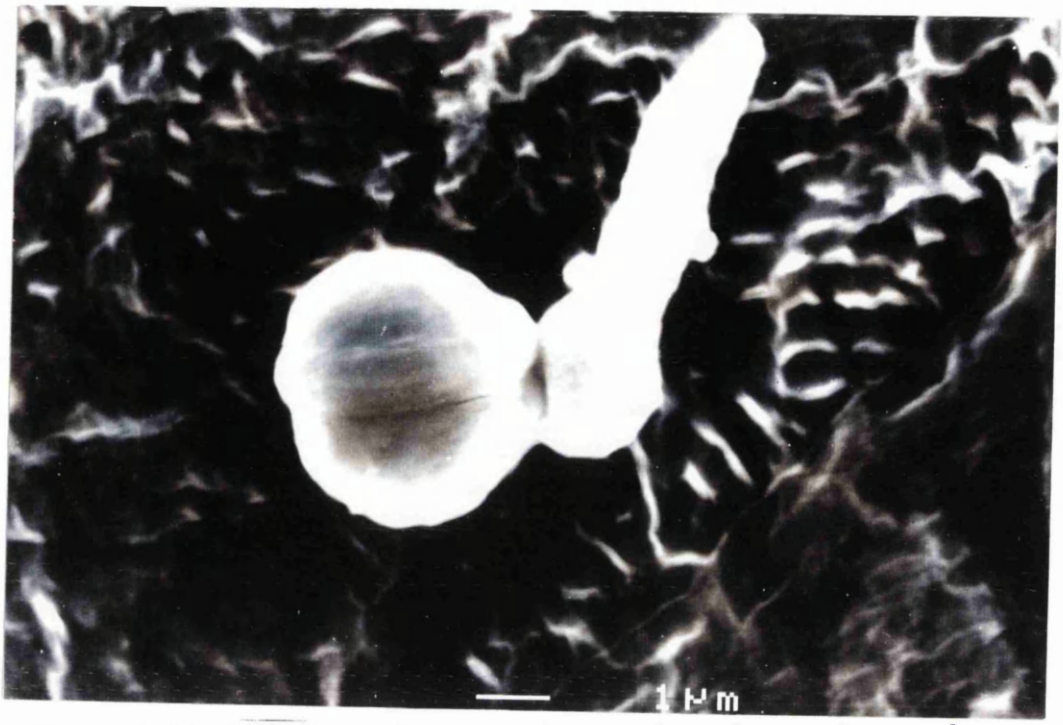


Figure 2:14 Scanning electron micrographs of germlings of *T. interdigitale* lying on the stratum corneum (a) An early germ tube after 16h of incubation. Note the appearance of side branches X 16000 (b) after 20h of incubation. Note the adherence of hyphal branch and secondary germling. The hyphal element appears to be partially embedded in the corneocyte X 4000 (c) a depression is formed in the corneocyte (arrow) where the filament is lying X 4400.

Figure 2:14b

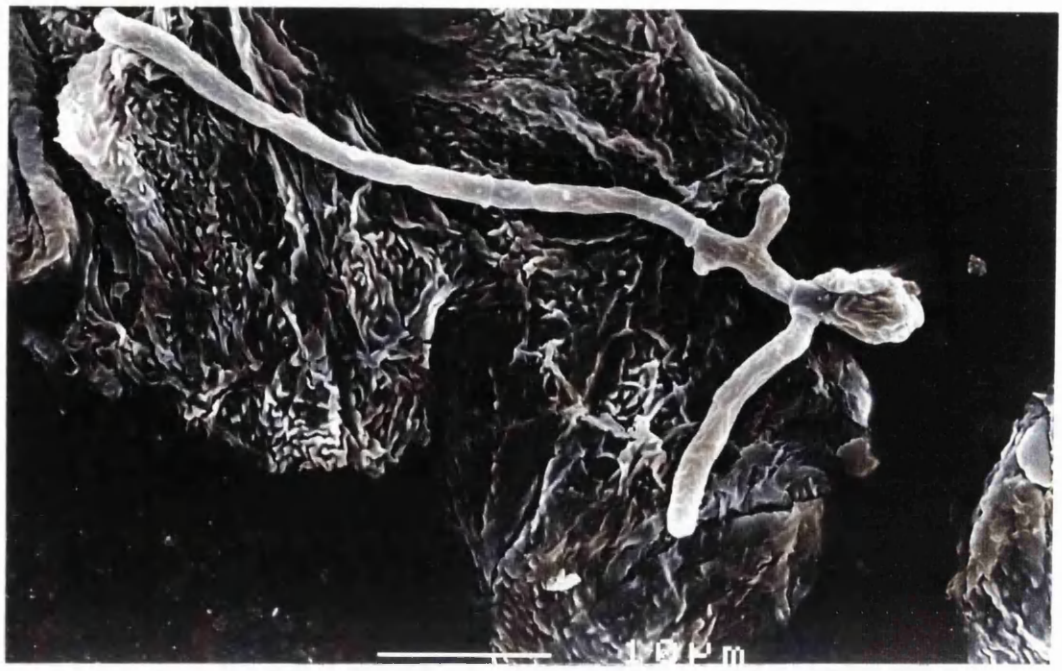


Figure 2:14c

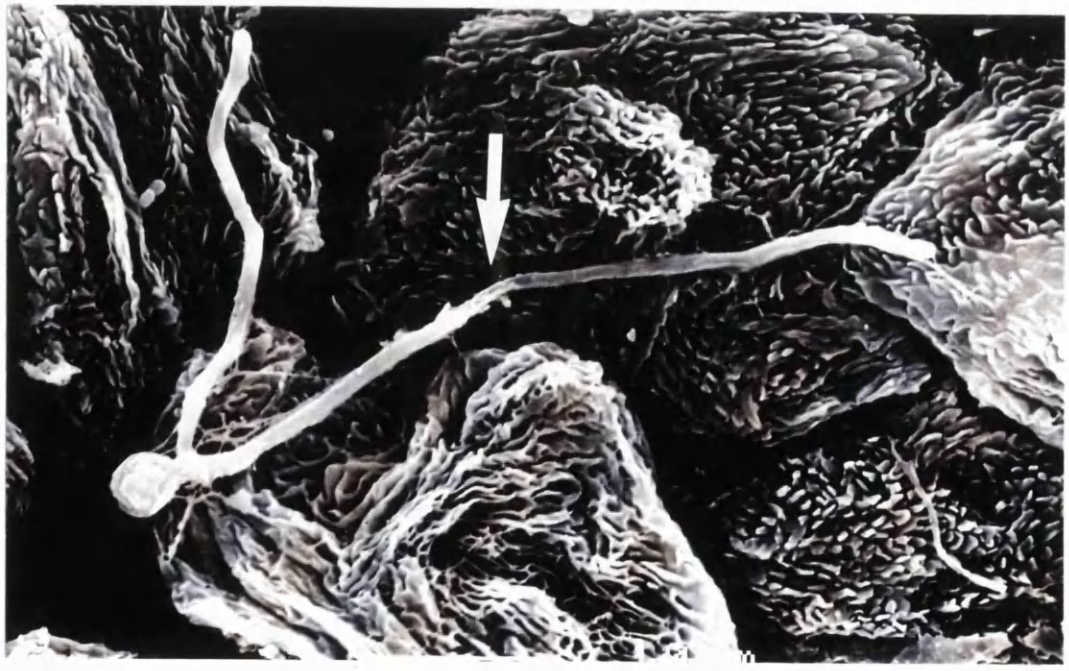


Figure 2:15a

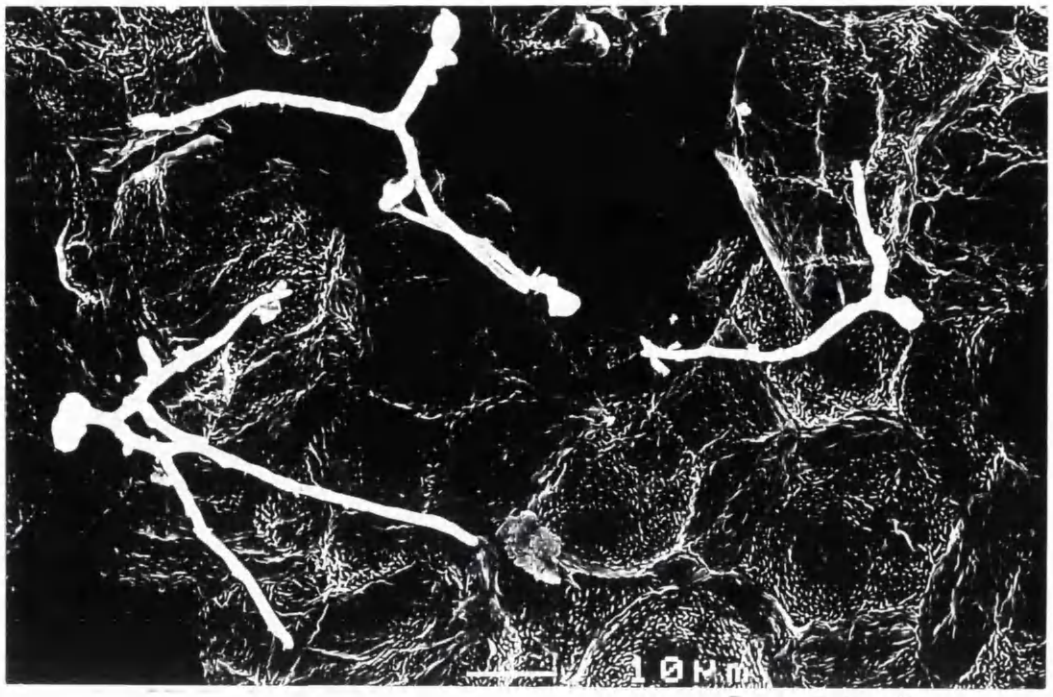


Figure 2:15b

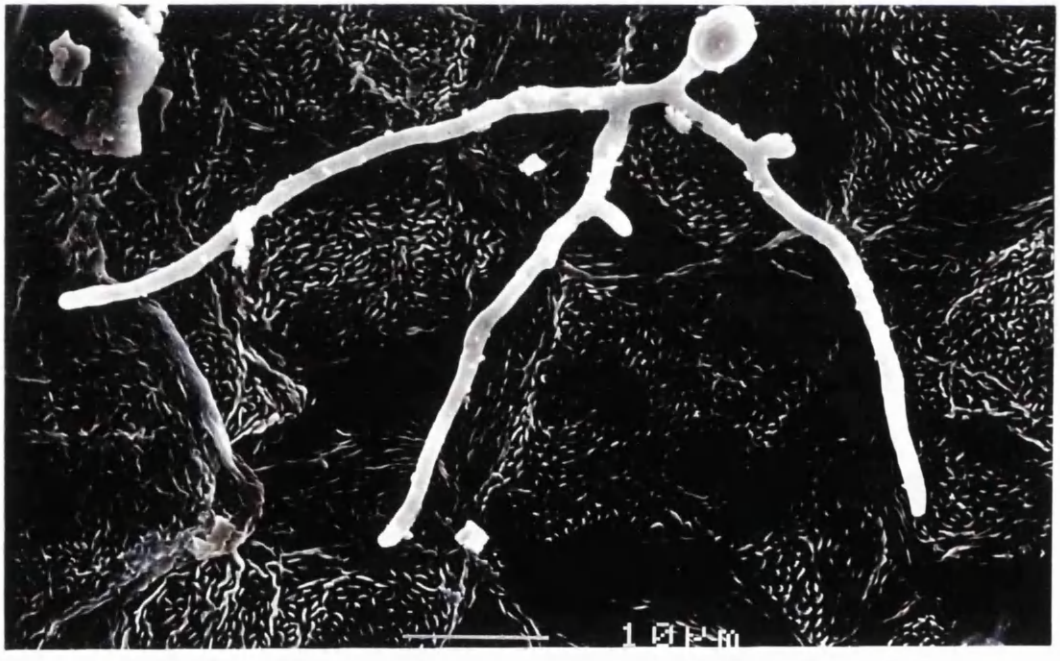


Figure 2:15 Scanning electron micrograph of *T. interdigitale* germlings lying on the stratum corneum after 20h incubation (a) Note the appearance of side branches and secondary germlings X 2000 (b) and the close relationship of the hyphal elements to the stratum corneum X 3400.

Figure 2:16a

Figure 2:16b

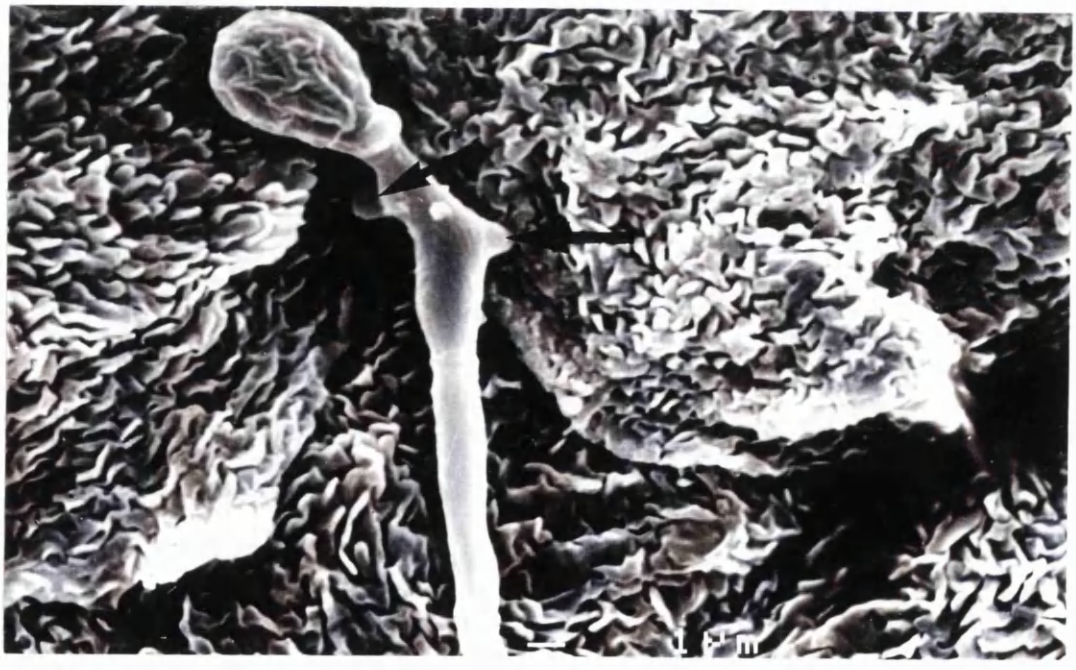


Figure 2:16 Scanning electron micrograph of *T. interdigitale* germling penetrating corneocytes (a) Note the germling appearing through the body of the corneocyte with side branches hanging downwards X 5400 (b) a germling lying in close apposition to corneocytes and its side branches are tending to penetrate through and between corneocytes (arrows) X 8000.

Figure 2:17a

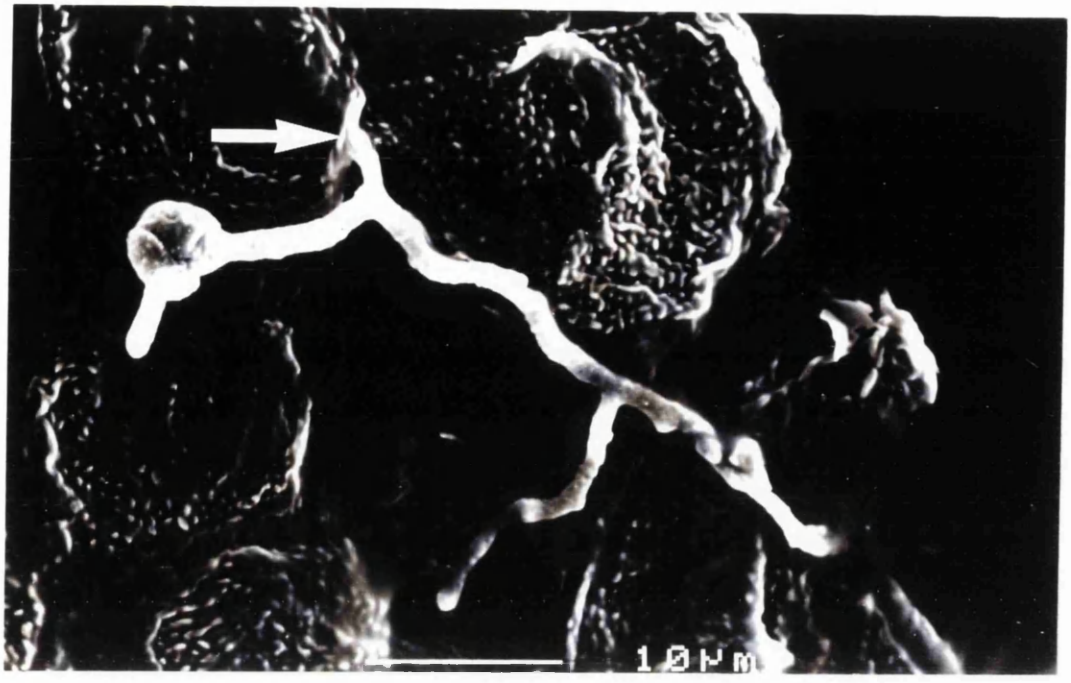


Figure 2:17b

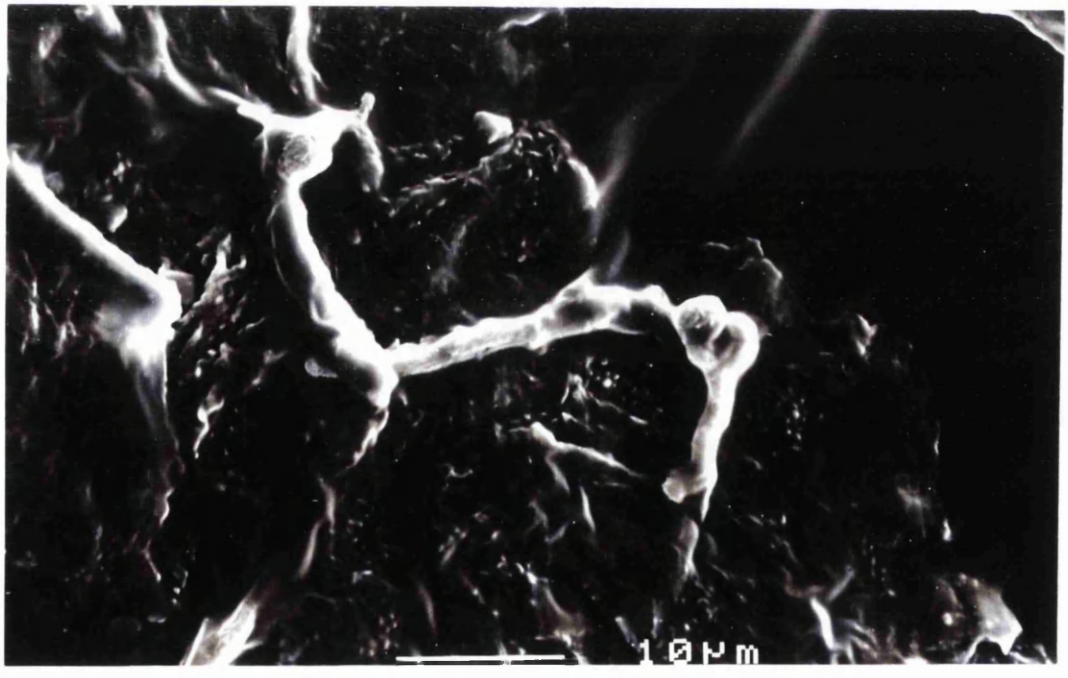


Figure 2:17 Scanning electron micrograph of stratum corneum inoculated with arthroconidia of *T. interdigitale* and incubated for 24h at 28°C (a) Note the germling extending over the surface of and attached to the margins of corneocytes. The side branches are seen to penetrate the corneocyte (arrow) and grow on bare areas X 5000 (b) a germling appears to be buried in the corneocyte producing a tunnel and ridges X 3800.

Figure 2:18a

Figure 2:18b

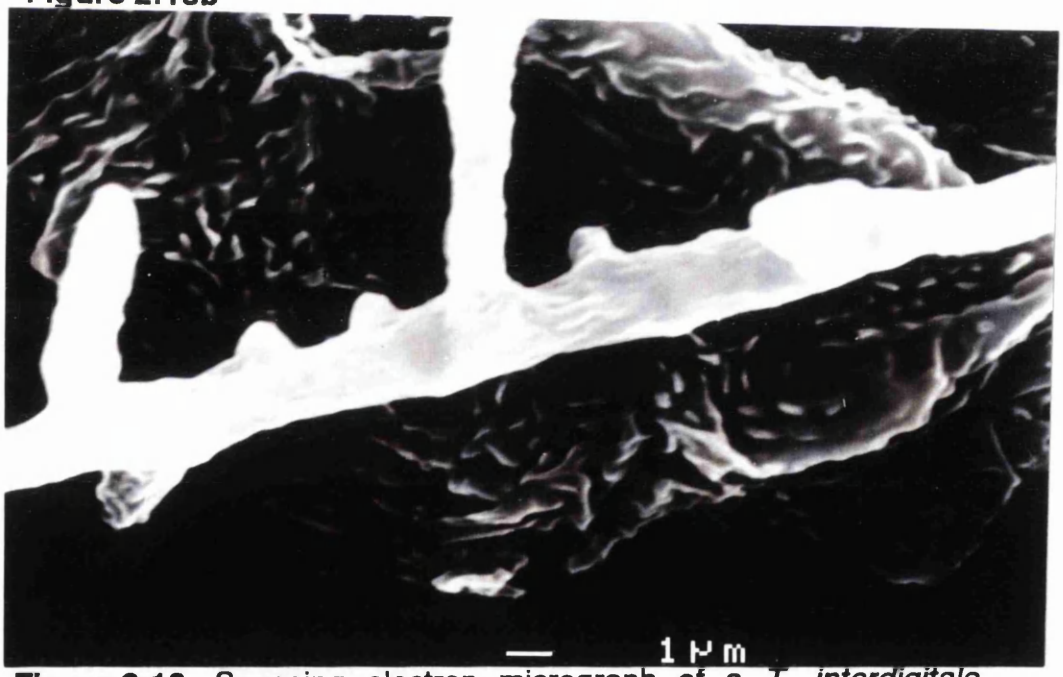


Figure 2:18 Scanning electron micrograph of a *T. interdigitale* mycelium on the stratum corneum after 24h incubation (a) Note the growth of side branches and secondary germlings in the direction of corneocytes X 900 (b) the tips of the side branches and secondary germlings arising are pointing in the direction of the body of the corneocyte X 10000.

Figure 2:19a

Figure 2:19b

Figure 2:19 (a) Scanning electron micrograph of stratum corneum stripped on D-SQUAME, and not inoculated with arthroconidia. More than one layer is present and the corneocytes appear normal X 4000 (b) Transmission electron micrograph showing the villi of corneocytes X 60000.

Figure 2:20 Scanning electron micrograph of arthroconidia inoculated on D–SQUAME without stratum corneum after 20h incubation at 28°C. Note the poor germination and clumping together of arthroconidia. The germ tubes are short with no side branches X 4600

2:6 Discussion

2:6:1 Germination of arthroconidia on different body surfaces

Arthroconidia started to germinate around 4h with a gradual increase in germination with further incubation to reach 100% on the face, and back of hand and in the range of 80–95% on the leg, palm and soles by 24h. Germination on the face was optimal and it was clear that no inhibitory effect of sebum was apparently evident on arthroconidial germination. Aljabre *et al.* (1992a) did not find any significant difference in germination of arthroconidia on stratum corneum from areas rich in sebaceous glands like cheek and upper back and areas such as palms and soles which were devoid of sebaceous glands. Kligman. (1963) did not find any inhibitory effect of sebum on fungal growth. Sebum contains 35–55% free fatty acids which are antifungal *in vitro* (Kligman, 1963). This property of fatty acids is utilized to treat dermatophyte lesions by undecylenic acid and its derivatives (Lyddon *et al.*, 1980). Alternatively, the amount of sebum in the stripped stratum corneum was not enough to interfere with the germination process.

Germination of arthroconidia is a virulence factor and rapid germination followed by hyphal extension and penetration of stratum corneum are pathogenic stages in the establishment of dermatophytosis (Richardson and Aljabre, 1993). Adherence of arthroconidia to corneocytes occurs first (Aljabre *et al*, 1993) but is not essential as adherent and non–adherent arthroconidia have been shown to germinate. Kimura and Pearsall (1980) demonstrated a strong correlation between germination and increased adherence of *C. albicans* to human epithelial cells and postulated that this enhanced adherence resulted from changes in the fungi that occur during germination. It is known that arthroconidia undergo swelling before the germ tubes emerge (Scott *et al.*, 1984), possibly as a result of intake of water and an increase in respiration and biosynthesis of cell components. However, the effect of these changes on

arthroconidial adherence to corneocytes is not understood (Aljabre *et al.*, 1993). Germination of *C. albicans* confers a heightened mechanical ability to resist phagocytosis (Davies and Denning, 1972) and it has been demonstrated that dermatophyte arthroconidia do not need an opsonin for ingestion and can be readily ingested by neutrophils (Richardson, 1990). It is possible that germination may confer arthroconidia the ability to resist phagocytosis. The data from the present study suggests that there appears to be a direct relationship between percent germination and hyphal extension on the face, back of hands and leg and an inverse relationship on palms and soles (Fig 2:3–4). Similarly, in *C. albicans* there is a clear relationship between the ability to form germ tubes and virulence (Richardson and Smith, 1981).

The various factors affecting germination of *T. mentagrophytes* arthroconidia *in vitro* have been extensively studied (Hashimoto and Blumenthal 1977; reviewed in Hashimoto, 1991) and similarities have been found between some of these factors and those present in the human cutaneous tissue However, the exact mechanism of arthroconidial germination *in vivo* is not known. Aljabre *et al.* (1992a) demonstrated that arthroconidia germinated readily in the presence of high humidity and stratum corneum, and showed only occasional germination in the absence of stratum corneum and postulated that factors necessary for germination may be present in corneocytes. In the present study, germination of arthroconidia and germ tube extension did occur in the absence of stratum corneum but was slow and irregular, and may be explained by the endogenous factors present in the arthroconidia. Some of the factors present in the skin which can enhance or induce germination and may account for the predilection of certain sites to infections by microorganisms may be due to the differences in composition of the stratum corneum and skin surface film (Burke *et al.*, 1966). Various amino acids are capable of inducing germination of *T. mentagrophytes* arthroconidia *in vitro* (Hashimoto and Blumenthal, 1977). Burke *et al.* (1966) described the free amino acid and water soluble peptide patterns of stratum corneum and showed that there was a

gradient of concentration in several amino acids from upper trunk to upper thigh to lower leg down to plantar surface. This could explain the difference in rates of germination of arthroconidia on different body surfaces and possibly the germ tube length. Amino acid-induced germination of arthroconidia occurs optimally at 35°C at pH 6-6.5 (Hashimoto, 1991) which bears a resemblance to the normal temperature and pH of the skin (Jolly *et al.*, 1960). The absence of corneocytes on control D-SQUAMES may explain the poor germination and growth of arthroconidia and germ tubes. The hyphae seen there were thin and branchless. The germination seen on these control D-SQUAMES and their further growth could be due to the germination requirements of the arthroconidia which are relatively non-stringent and they can germinate in the presence of limited nutrients and also when no exogenous nutrient source is present. This suggests that these spores may possess endogenous sources of nutrients which enable them to germinate and form germ tubes.

The exact stimuli involved in producing arthroconidial germination on the stratum corneum are not understood. Keratinocytes on direct activation are capable of producing the complete repertoire of proinflammatory cytokines and initiate inflammation (Barker *et al.*, 1991). It is possible that arthroconidia in close proximity to corneocytes initiate signals which can further initiate the process of germination. The contact between dermatophyte arthroconidia and corneocytes is very close, with the presence of a fibrillar-floccular material between the two cell types (Aljabre *et al.*, 1993; Rashid *et al.*, 1993). The exact nature of this material is unknown. This material appears to be evenly distributed on the arthroconidial cell surface (Aljabre *et al.*, 1993) and penetrates into the crevices of the corneocytes (Rashid *et al.*, 1993). Changes have been noticed in the corneocytes which increase their arthroconidia-binding capacity. Microvilli of corneocytes are thought to help in the initial entrapment of arthroconidia (Aljabre *et al.*, 1993). Where adherence was seen, the crenated appearance of the corneocyte membrane was absent and a smooth attachment area was evident with a slight depression (Rashid *et al.*,

1993). No apparent damage to corneocytes was seen. Multiple side branches arising from the germ tubes and the hyphal tip were seen to be growing in a directional way towards adjoining corneocytes suggesting that the factors for their growth were arising in the corneocytes.

Signal transduction pathways in keratinocytes are present. Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) have been associated with keratinocyte proliferation and differentiation (Rosenbach and Czarnetzki, 1992). cAMP cascade is involved in the control of dormancy and induction of germination in fungal spore (Thevelein, 1984) and it is possible that it is involved in inducing arthroconidial germination on stratum corneum.

On electron microscopy, damage to corneocytes was observed by hyphae during the penetration of stratum corneum and similar changes were also observed by Aljabre *et al.*, (1992a). It is possible that arthroconidia on adhering to corneocytes initiate signals which can then further initiate the process of cutaneous inflammation. Alternatively, damage to corneocytes could lead to the release of cytokines and inflammation. A common sequel to inflammation is epidermal hyperproliferation as manifested by thickened skin and scale formation (Barker *et al.*, 1991). It has been observed in experimental dermatophytosis that there is a rapid epidermopoiesis which helps to eradicate skin infection (Tagami, 1985). IL-1 is known to induce proliferation of keratinocytes, and the normal human stratum corneum contains a large amount of IL-1, the significance of which is not known. It has been shown that disruption of the skin causes prompt release of IL-1 leading to host defense responses (Nozaki *et al.*, 1991). The dermatophyte arthroconidia have to overcome these factors by rapid germination and penetration of the stratum corneum by germ tubes. It was observed that by 24h, germ tubes had penetrated the stratum corneum and were lying insinuated in the layers of corneocytes.

2:6:2 Germ tube extension on different body surfaces

Hyphal growth was maximal on face and back of hands and was not comparable to growth on leg, palm and soles. Germlings were observed in close apposition to the corneocytes. After an initial phase of 16h of linear growth, there followed a phase of exponential growth of the germ tubes with side branches and secondary germlings appearing and intermingling and peripheral expansion of the mycelium. Fungal spores can demonstrate a linear and exponential growth phase (Trinci, 1971). Smith (1924) demonstrated exponential growth of fungal hyphae on solid medium after a initial phase of linear growth. Variation between the length of different hyphae on the same site was observed and reflected the periodic slowing of the growth rate of some leading hyphae and the concomitant acceleration of growth of some hyphae behind the apex. This has been described for many observations of hyphal growth (Smith, 1924). The initial linear growth of germ tubes on the stratum corneum can be explained by the fact that endogenous reserves within arthroconidia support it while the later growth is supported by the stratum corneum. The formation of a vegetative mycelium on different body surfaces follows the same rules as those for the development of a fungal colony from the point of inoculation (Trinci, 1974). It was seen that branches were arising at intervals from the hyphae, mostly being subapical although apical branching was rarely seen and secondary germlings were observed. The regular formation of hyphal branches accounts for the exponential growth of the dermatophytes on stratum corneum. The exact mechanisms underlying branching in filamentous fungi are not known. Ionic currents seem to play a crucial role in the polarity and branching in fungi. It has been seen that currents precede hyphal branch formation . These currents are directed into the trunk of hyphae at sites where the branches will emerge (Gow, 1989). Another factor in hyphal branching is wall lysis and it is thought that the expansion of the hyphal tip occurs by wall synthesis which is in delicate balance with wall lysis (Gow,

1989). Cyclic AMP has been implicated in the regulation of hyphal growth. Branching and spore germination in fungi and basal levels of cAMP are necessary for normal mycelial development (Robson, 1991). Cyclic nucleotides may influence the dermatophyte morphology on the stratum corneum by activation or deactivating enzymes involved in tip wall growth or branch initiation. By not swabbing the body surfaces with antiseptics and no prior antifungal medication used, the dermatophyte–bacterial interaction was allowed to occur in an environment similar to the *in vivo* situation. Dermatophyte fungi are known to produce antibiotics which can suppress the resident flora and thus assist in the establishment of dermatophyte infection (Youssef *et al.*, 1978). Furthermore, bacteria may secrete specific chemotactic agents for the directional growth of hyphae (Bibel and Smiljanic, 1979).

Growth of the germ tubes was slower on the palms and soles and a drop in germ tubes length was observed after 20h (Fig 2:4). The decrease in hyphal length on the palm and soles could be due to the combination of a number of factors. The stratum corneum on the palms and soles is much thicker and more permeable than stratum corneum on other body sites and it is possible that factors inhibitory to the growth of dermatophytes may be able to diffuse onto the surface and inhibit growth. Variations have been observed in corneocytes with the anatomic body areas, age, sex and seasons. Similarly, corneocytes vary in surface area and thickness with body region (Plewig and Marples, 1970). These differences could possibly explain the difference in growth rate of germ tubes on different body surfaces. Hyphae of *T. mentagrophytes* have been observed to penetrate through corneocytes and subsequently damaging them. The corneocytes are covered by a thick cornified cell envelope and plasma membrane. Richardson and Aljabre, (1993) have postulated that this could be due to a combined chemical and mechanical effect of the hyphae. As the stratum corneum is thicker on the palms and soles it may be difficult for the hyphae to penetrate the layers at the same rate as on other body surfaces.

In addition Baer *et al.* (1955) described that the foot skin of most people had a remarkable capacity to rapidly rid itself of the inhibiting pathogenic fungi. Rosenthal and Baer, (1966) attempted to induce tinea pedis by immersing feet of volunteers in suspensions of dermatophytes and were unable to produce a single case of acute tinea pedis and concluded that mere exposure of healthy feet to fungi was not sufficient to induce clinical disease. Feet which had blistering trauma produced prior to inoculation of the fungus did show evidence of experimental infection.

2:6:3 Effect of temperature and humidity on the germination of arthroconidia on the stratum corneum

In the present study no arthroconidial germination was seen at 4°C, 45°C and in the absence of moisture. These substantiate the findings of Aljabre *et al.*, (1992a). Emyanitoff and Hashimoto (1979) showed that the temperature range for arthroconidial germination in the presence of simple amino acids was 20–39°C, optimal 37°C. Aljabre *et al.* (1992a) did not find any significant difference in the germination rate of arthroconidia on the stratum corneum at 28°C or 37°C. They did not observe any germination of arthroconidia at 4°C or 45°C and in the absence of moisture. Knight (1976) found the optimal temperature range for the growth of *T. mentagrophytes* spores on human stratum corneum *in vitro* to be between 27 and 33°C. Paldrok (1955) found that the temperature for best vegetative growth to occur and the optimal temperature range for *T. mentagrophytes* was between 27 and 33°C.

2:7 Conclusion

In this study, the growth of dermatophytes on different body surfaces was observed. The data suggests that there is an apparent relationship between germination of arthroconidia and hyphal elongation on certain body

surfaces. The sequence of events involved in the formation of a vegetative mycelium on different body surfaces studied, showed that there was a close relationship between arthroconidia and germ tubes and the corneocytes. The close apposition between arthroconidia and corneocytes and directional growth of hyphae and side branches towards adjoining corneocytes and the poor growth of arthroconidia in the absence of stratum corneum point to the crucial role of corneocytes in the growth of dermatophytes on body surfaces. Previous studies have emphasised the importance of skin hydration in the establishment of lesions and that maceration and occlusion encourage infection. In this study dermatophyte infection was established in the absence of these factors using dermatophyte arthroconidia. It seems that the dermatophyte used in the present study had an enhanced ability to establish infection and overcome some of the resistance factors of the skin. The D-SQUAME model used provided a simple, rapid and inexpensive method for the study of biology of dermatophyte infections.

Chapter 3

Activity of terbinafine in the stratum corneum on dermatophyte fungi

3:1 Summary

The application of stripped sheets of stratum corneum as a model to assess the effects of antifungal drugs on dermatophytes was investigated. A strain of *T. interdigitale* and therapeutic concentrations of terbinafine were used. The effect of terbinafine incorporated into stratum corneum from different body surfaces on the germination of arthroconidia and germ tube elongation was assessed by light microscopy. The morphological modifications induced by terbinafine in the arthroconidia and germ tubes were observed by electron microscopy. Inhibition of germination and germ tube length was observed on different body surfaces examined. Pores and erosions were present in the cell wall and cell membrane of arthroconidia. Dilated vacuoles were observed and small electron dense areas were evident. Inhibition of germ tube length occurred and hence true mycelia were not formed. Germ tubes showed pores along their length. Germ tubes and side branches which had formed were observed to have collapsed at higher drug concentrations. This study suggests that dormant arthroconidia are susceptible to the therapeutic concentration (6.2–100 mg/L) of terbinafine with subsequent fungicidal alterations to the cytosol and intracellular organelles. By reducing germ tube elongation, terbinafine could abrogate the pathogenic ability of dermatophytes to establish infection.

3:2 Introduction

Development of new antifungal agents is markedly hampered by a lack of alternative fungus-specific test methods *in vitro* and a limited number of reproducible infection models *in vivo* (Rinaldi, 1987). The routine test methods used to study antimycotic drugs *in vitro* include: broth dilution, agar dilution and agar diffusion (Warnock, 1992). However, the current applicability of antifungal

susceptibility tests is limited by inadequate standardisation and insufficient correlation of test results to clinical usage (Galgiani, 1987).

Germination of arthroconidia and hyphal penetration of the stratum corneum are recognised pathogenic factors in dermatophytosis (Richardson and Aljabre, 1993). For an antifungal agent to be effective in dermatophyte infections it must be able to inhibit these two parameters of fungal growth hence affecting colonization of the stratum corneum. Little information is available on how antifungal drugs inhibit germination and hyphal extension.

Inocula used for *in vitro* testing of antifungal drugs consist of mycelial preparations of varying purity. Arthroconidia are the main infectious propagules of dermatophytes and the only parasitic conidia found in tissues (Hashimoto, 1991). Recurrences of dermatophyte infections have been attributed to the activation of dormant arthroconidia under suitable conditions (Hashimoto and Blumenthal, 1977; Aljabre *et al.*, 1992a). These conidia have rarely been used in sensitivity testing of antifungal agents mainly because of the problem of producing pure arthroconidial inocula (Wright *et al.*, 1983; Scott *et al.*, 1984). Such tests were conducted in laboratory media. The growth of dermatophytes on solid culture media is not comparable to their growth on the stratum corneum *in vivo* or *in vitro* (Rashid *et al.*, 1993). Moreover, obfuscation of the activity of antifungal agents by culture media has been seen (Hoeprich and Finn, 1972). Therefore, it is not appropriate to assay antifungal drug activity on solid media and compare it with drug activity on the stratum corneum.

To examine fully the effect of an antifungal agent on a pathogenic fungus, it is essential to expose the appropriate morphological form(s) to therapeutic concentrations of an agent whilst it is growing on its natural substrate (Rashid *et al.*, 1993). Growth of dermatophytes may be compared on untreated or normal stratum corneum previously exposed to a antifungal drug (Knight, 1973b). It is possible to assay antifungal activity within the stratum corneum by culturing dermatophyte arthroconidia on sheets of stratum corneum

(Rashid *et al.*, 1993). Aljabre *et al.* (1991) used human corneocytes as an *in vitro* model to study antifungal drugs.

Terbinafine is currently the most interesting antifungal agent in use for the treatment of dermatophyte infections. It has a unique mode of action contributing to its fungicidal action (Ryder and Mieth, 1992). Although it has a broad spectrum of antifungal activity, dermatophytes are the most sensitive organisms to this drug (Ryder and Mieth, 1992; Shear *et al.*, 1992) and additionally arthroconidia and not micro or macroconidia are the most relevant infectious particles of these fungi and were therefore selected for this study. Since the target tissue of dermatophytes is the stratum corneum and terbinafine along with other antifungal drugs concentrates in the stratum corneum (Artis, 1985; Faergemann *et al.*, 1990; Lever *et al.*, 1990), the stratum corneum model was selected as it has a close resemblance to the *in vivo* environment.

3:3 Aim of the study

This part of the study was undertaken to investigate the effects of therapeutic concentrations of terbinafine on the germinative ability of the arthroconidia to germinate and to assess the inhibitory effect on germ tube elongation by *T. interdigitale*. In addition the morphological and ultrastructural changes in arthroconidia and germ tubes after exposure to terbinafine whilst growing on the stratum corneum were examined by scanning and transmission electron microscopy. The possibility of application of the stratum corneum was explored as a *in vitro* antifungal assessment test method.

3:4 Materials and methods

3:4:1 Organism and stock cultures

T. interdigitale strain described in Chapter 2, section 2:4:1 was used in this study. Stock cultures were maintained as described in Chapter 2, section 2:4:2.

3:4:2 Production and preparation of arthroconidia

Arthroconidia were produced and prepared to a concentration of 5×10^4 arthroconidia per ml as described in Chapter 2, sections 2:4:3 and 2:4:4.

3:4:3 Preparation of stratum corneum sheets

Sheets of stratum corneum were prepared as described in Chapter 2, section 2:4:5.

3:4:4 Preparation of terbinafine solutions

Terbinafine (batch No. 90C252) was a generous gift from Sandoz Pharmaceutical, UK. Stock solutions of the drug were prepared by the method described by Warnock (1989). 50mg of terbinafine was dissolved in 5ml of dimethyl sulphoxide (DMSO) and allowed to stand for 30 mins to permit self-sterilisation, then dispersed in 1ml volumes and stored at 4°C until use. To 1ml of the drug solution was added 9ml of sterile distilled water to achieve a 1000 mg/L stock solution. Appropriate volumes of stock solution were added to distilled water to obtain a concentration range of 6.2 to 100 mg/L.

3:4:5 Assessment of antifungal activity

Stratum corneum sheets were saturated with terbinafine by exposing the adhesive side of a D-SQUAME disc with attached skin cells to therapeutic concentrations (6.2 - 100 mg/L) of the drug for 15 mins and 3h and then removed from the drug solutions and placed on a microscope slide in a plastic dish. For control purposes D-SQUAME discs with skin cells were exposed to DMSO and distilled water in an equivalent concentration to that of final drug concentration and incubated for the same period of time. The discs were then point inoculated with 50μl of a $5x10^4$ arthroconidia per ml suspension so that 5-10 cells were seen at x400 magnification and placed in a humidified atmosphere and then incubated at 28°C for 20h. Two parameters were used to assess the antifungal activity of the drug:

3:4:5:1 Effect of terbinafine on the germination of arthroconidia on different body surfaces

This consisted of determining percent germination of arthroconidia by counting the number of germinated arthroconidia at 20h after being exposed to the drug.

3:4:5:2 Effect of terbinafine on the germ tube extension on different body surfaces

This consisted of measuring the inhibitory effect of therapeutic concentrations of terbinafine on germ tube elongation by means of a graduated microscope eyepiece after 20h at 28°C.

3:4:6 Effect of terbinafine on the morphology and ultrastructure of arthroconidia and germ tubes

Strips of stratum corneum were saturated with terbinafine and then inoculated with ungerminated arthroconidia of *T. interdigitale*. The stratum corneum sheets were incubated at 28°C for 20h and then fixed in formaldehyde. Samples were then stained by Periodic Acid Schiff (PAS) stain (Appendix).

For electron microscopy, strips of stratum corneum were saturated with terbinafine and then inoculated with ungerminated arthroconidia of *T. interdigitale*. The stratum corneum sheets were incubated at 28°C for 16, 20, 24h and then fixed in 3% (v/v) glutaraldehyde in a 0.1M sodium cacodylate buffer and processed for electron microscopy as described in Chapter 2, section 2:4:9.

3:5 Results

3:5:1 Effect of terbinafine on the germination of arthroconidia on different body surfaces

After 20h incubation, arthroconidial germination was seen in distilled water (control) as well as in the presence of terbinafine on different body surfaces. On the face (Fig 3:1a) 15min exposure to 6.2 mg/L caused an initial drop in germination followed by stabilisation of the germinative ability at 100 mg/L. At 100 mg/L concentration of terbinafine no apparent difference was observed in the germination rate of arthroconidia on the face. Fig 3:2a shows the results from the back of the hand. Following 15 min exposure there was no obvious drop in germination even at higher drug concentrations (100 mg/L) however after 3h there was a 50% drop in the germination. On the palm (Fig 3:3a) 15 min exposure an initial increase in germination rate gradually reaching

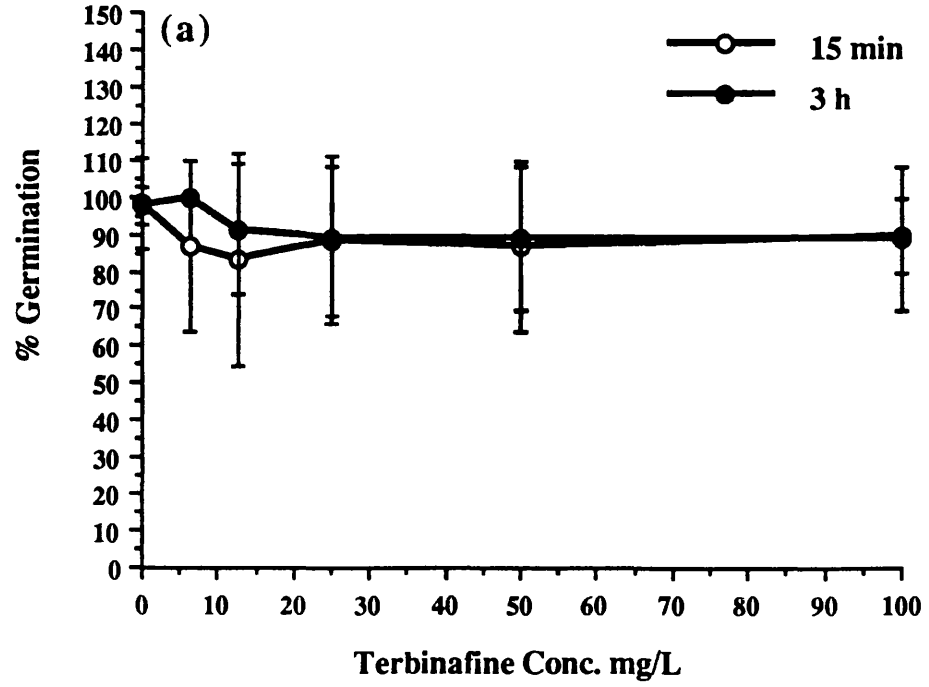
to control levels at 100 mg/L. While the longer exposure of 3h showed a similar initial rise in germination which reached below control level at 100 mg/L. On the leg (Fig 3:4a) 15 min exposure gave an initial drop in germination at lower concentrations 6.2 mg/L of the drug which was followed by stabilisation of the germination rate at 100 mg/L drug concentration. At 3h the arthroconidia were not clearly visible. On the sole (Fig 3:5a) a marked drop in the germination rate was observed following 15 min exposure. Again at 3h readings were not taken since at 20h arthroconidia could not be clearly quantified.

Taken together the readings suggest wide variation in the response of the arthroconidia to the various drug concentrations on the different body surfaces. On the face, palm and leg no apparent inhibitory effect was observed on arthroconidial germination even at high drug concentrations while on the back of hand and sole more than a 50% drop in germination was observed at high drug concentrations.

On all the sites examined there was a tendency towards an initial drop in the ability of the arthroconidia to germinate at lower drug concentrations which appears to be followed by a gradual normalisation of the germination rate. Moreover, there was no apparent difference in the inhibition of germination between 15 min and 3h exposure except on the back of the hand.

3:5:2 Effect of terbinafine on germ tube extension on different body surfaces

Based on the findings in Chapter 2, section 2:5:1, and the fact that the fungicidal effect of terbinafine started to appear after 20h incubation, it was thought appropriate to measure the germ tube length after 20h incubation following exposure to therapeutic concentrations of terbinafine. After 20h incubation germ tube elongation was seen under the control conditions, while in the presence of terbinafine hyphal elongation was observed to be inhibited.



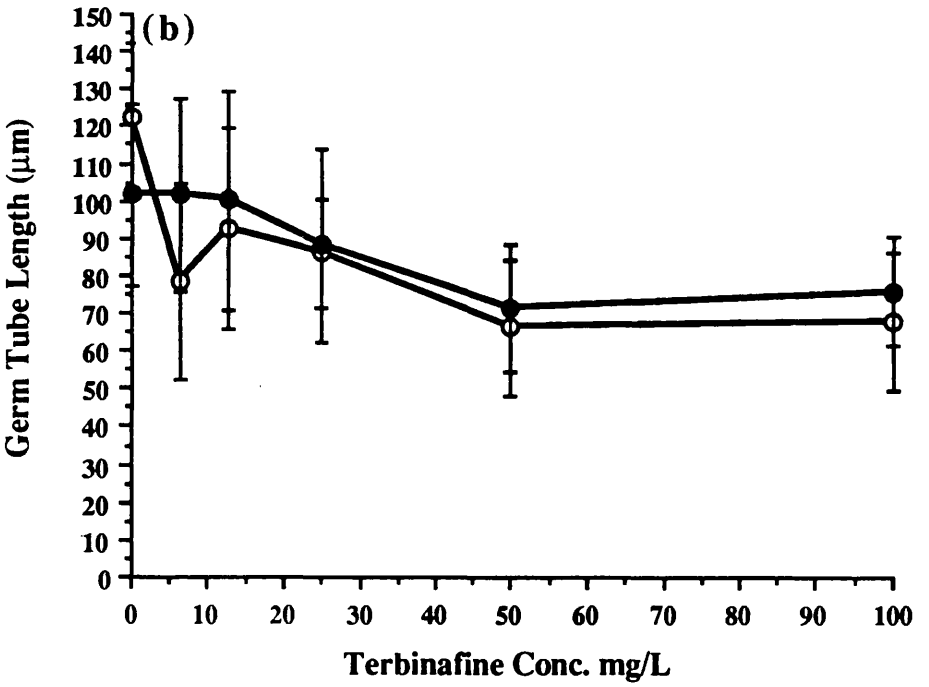
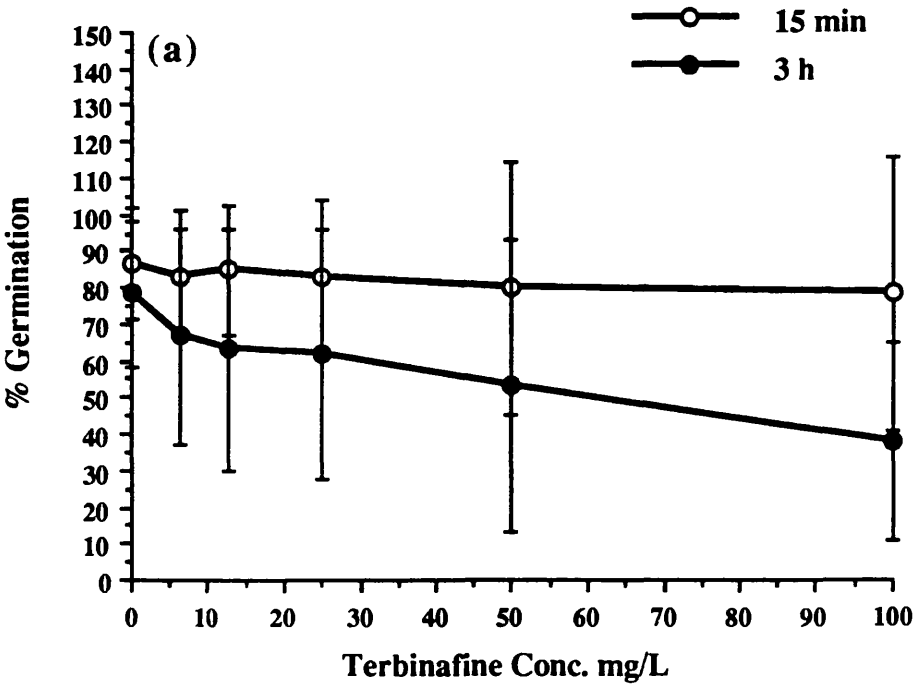


Figure 3:1 Effect of terbinafine concentration and exposure time on the % germination of arthroconidia (a) and germ tube length (b) on the face.

The counting was done in triplicate in three separate cultures and the values expressed as the average mean ± SD



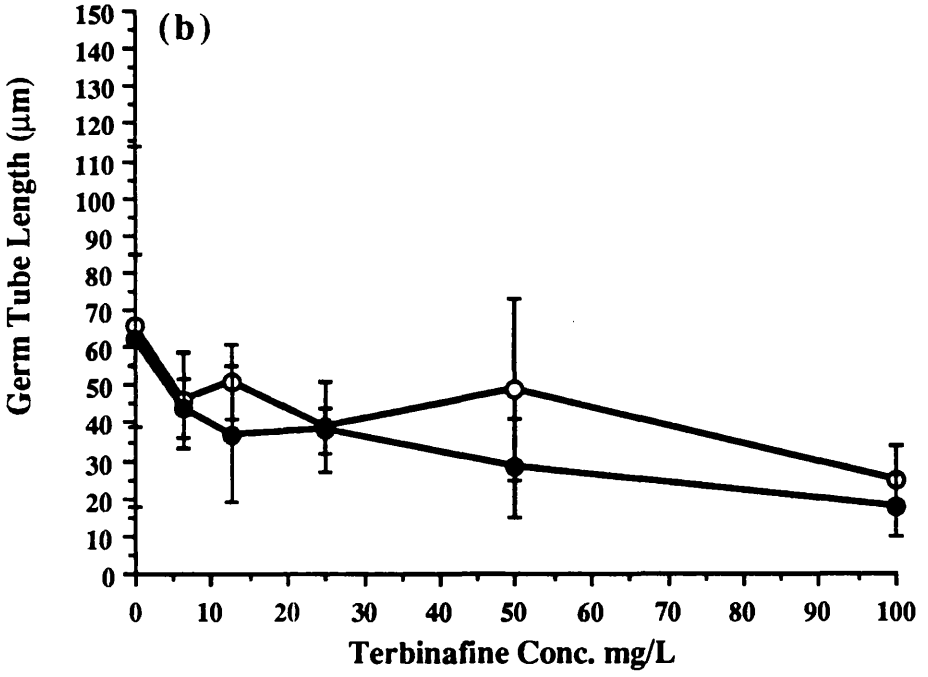


Figure 3:2 Effect of terbinafine concentration and exposure time on the % germination of arthroconidia (a) and germ tube length (b) on back of hand.

The counting was done in triplicate in three separate cultures and the values expressed as the average mean ± SD

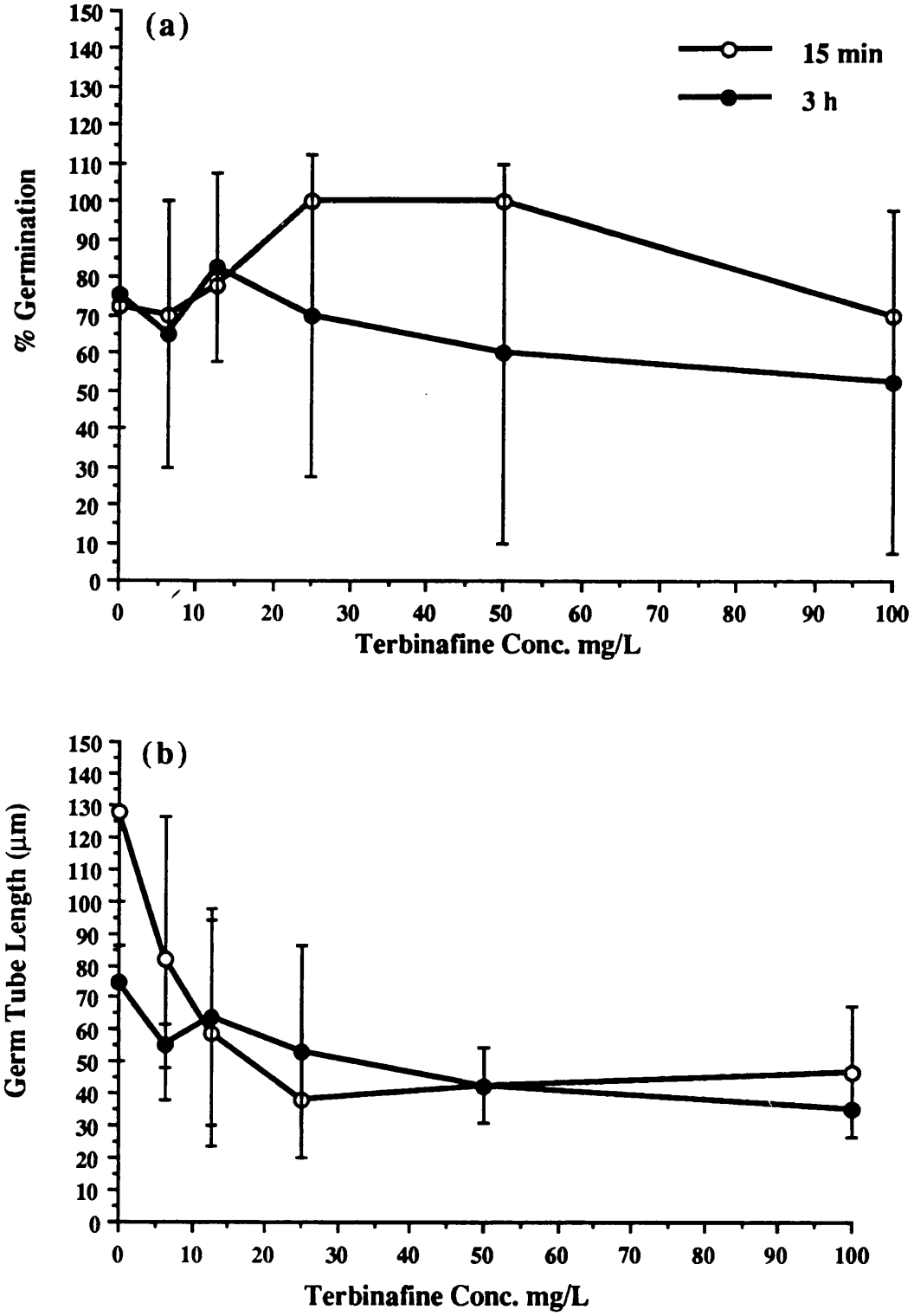
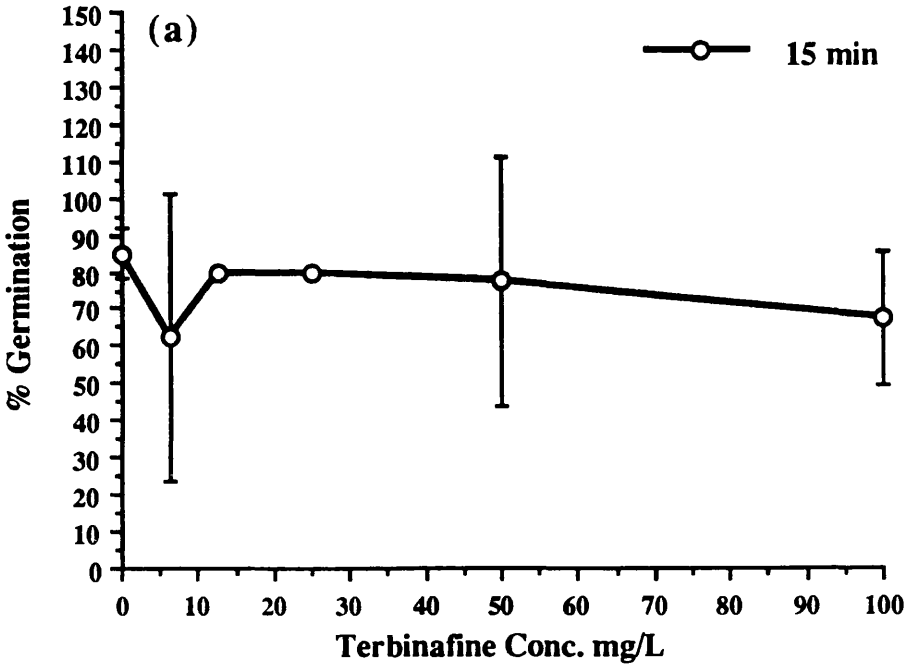


Figure 3:3 Effect of terbinafine concentration and exposure time on the % germination of arthroconidia (a) and germ tube length (b) on the palm.
The counting was done in triplicate in three separate cultures and the values expressed as the average mean ± SD



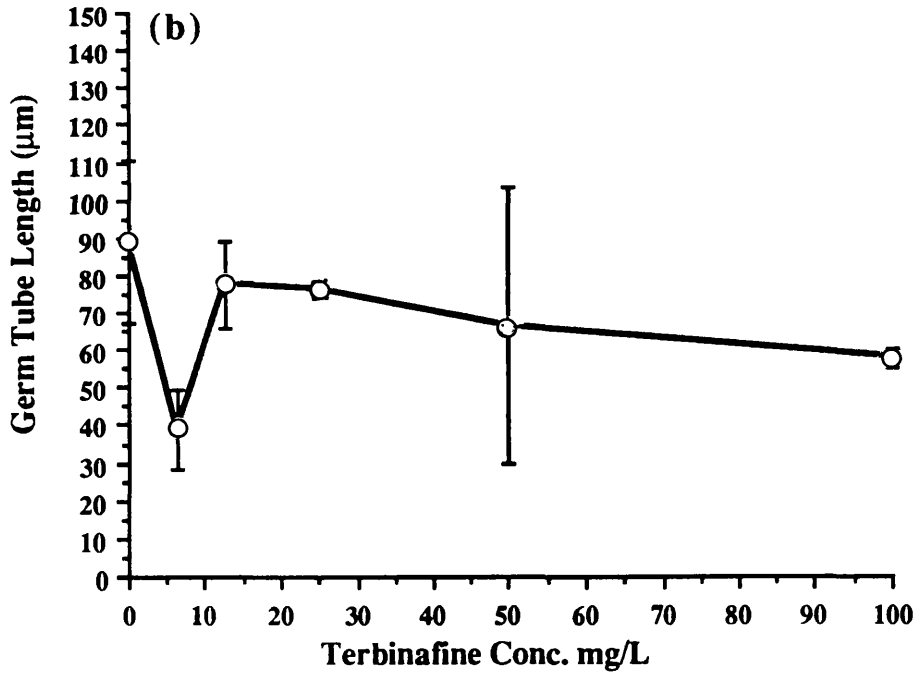
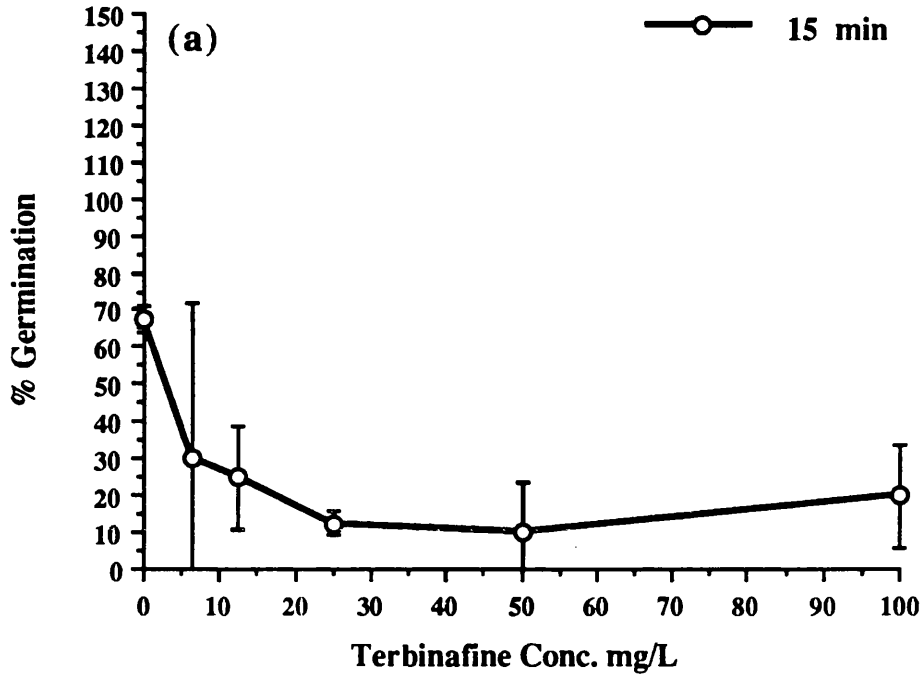


Figure 3:4 Effect of terbinafine concentration and exposure time on the % germination of arthroconidia (a) and germ tube length (b) on the leg.

The counting was done in triplicate in three separate cultures and the values expressed as the average mean ± SD



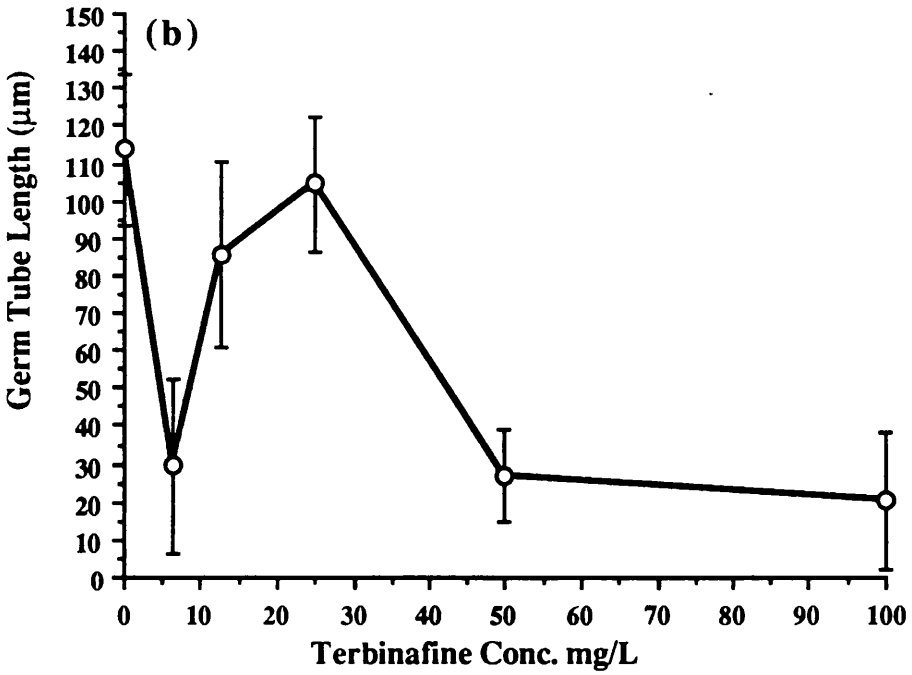


Figure 3:5 Effect of terbinafine concentration and exposure time on the % germination of arthroconidia (a) and germ tube length (b) on the sole.

The counting was done in triplicate in three separate cultures and the values expressed as the average mean ± SD

On the face (Fig 3:1b) a decrease in germ tube length was observed following 15 min and 3h exposure. The drop in germ tube lengths was more marked in the low drug concentration range but gradually reached near equal levels around 100 mg/L. Fig 3:2b shows the germ tube length on the back of the hand. There was an apparent drop in germ tube length following 15 min and 3h exposure. The drop in length was acute in the lower dose range and there was a greater than 50% inhibition of the germ tube length at 100 mg/L. Similar observations were made on palm and are shown as Fig 3:3b. A marked decrease in germ tube length was observed following exposure to terbinafine. The drop in length was acute after 15 min exposure followed by gradual drop (3h exposure). On the leg there was an initial decrease in germ tube length and reaching to below controls at 100 mg/L (Fig 3:4b). On the sole (Fig 3:5b) following an initial acute drop in germ tube length there was a sudden rise in length which then gradually decreased to marked levels at 100 mg/L. Due to poor clarity of specimen details at 3h no observations could be recorded in cases of leg and sole samples.

Variation in the germ tube length observed in response to various drug concentrations revealed an apparent decrease in tube length in response to the increasing drug concentrations on face, back of hand, palm, sole, while compared to decrease in length on the leg. There was a general tendency of reduction in the germ tube length following exposure to low concentrations of terbinafine on all body sites examined. A tendency towards more acute and greater drop in germ tube length was noted following 15 min exposure.

3:5:3 Morphological changes induced in arthroconidia and germ tubes following exposure to terbinafine

Control samples which were incubated for 16h showed well formed germ tubes with side branches. After 20h incubation, the germ tubes were longer and extended further with multiple side branches. By 24h, small microcolonies

had formed and by 48h vegetative mycelium was observed on all body sites examined (Fig 3:6). After 5 days incubation, the hyphae on the stratum corneum showed evidence of segmentation and arthroconidia formation.

In samples exposed to low concentrations of terbinafine, that is 0.25–2 mg/L, there was no visible inhibitory effect on germination, hyphal growth or the formation of mycelium. 100% germination was observed and a well formed mycelium was in place as was evident in the controls. When used at 4–8 mg/L an inhibitory effect of the drug was observed. Dilated hyphae with swollen ends were seen. The fungistatic effect of the drug was clearly seen at 4 mg/L and at 6.2 mg/L there was a clear fungicidal effect. The fungistatic effect appeared with the hyphae arrested in growth and no further extension taking place (Fig 3:7). The fungicidal effect of terbinafine was dose related and more marked at higher concentrations. While the fungicidal effect of the drug was becoming apparent a sequence of changes were observed in the arthroconidia and germ tubes . The changes were observed at 6.2 mg/L and above. The first sign was an arrest in germ tube extension followed by a lighter appearance of the arthroconidia and germlings on the background of the stratum corneum. This was followed by the outline of the walls becoming indistinct and an overall damaged appearance of the germ tubes as well as the appearance of side branches (Fig 3:8). These changes began to appear after 20h incubation, were marked at 24h and by 40–72 h only remnants of the germ tubes could be seen. No mycelium was observed at an concentration greater than 4 mg/L. A similar pattern of changes were observed on all body sites examined. The fungicidal effect was dose dependent and its onset more rapid at doses of 50 and 100 mg/L while it was gradual at doses between 6.2–12.5 mg/L. On light microscopy, the arthroconidia appeared spherical. Also an increase in their size was observed before germination. Swollen, ungerminated arthroconidia were also observed. Close apposition of the arthroconidia to corneocytes was observed. The corneocytes exposed to terbinafine appeared to be healthy.

Gaps were seen between corneocytes removed from palm and sole while uniform layers were removed from the other sites.

3:5:4 Effect of terbinafine on the ultrastructure of arthroconidia and germ tubes whilst growing on the stratum corneum

Scanning electron microscopy of samples after 16h incubation revealed that arthroconidia appeared as oval, spherical or cylindrical forms, often covered with remnants of the outer hyphal wall. This is described in detail in Chapter 2, section 2:5:2. In contrast, modifications to the gross morphology of the arthroconidia appeared in the presence of 6.2 mg/L terbinafine, although this effect was variable. These effects were more marked at drug concentrations of 50 and 100 mg/L. The arthroconidia became swollen and cell wall deformities and damage was concomitant with the inability of arthroconidia to germinate. Germination was partially inhibited by the concentrations of terbinafine used here. There was a variable effect on the arthroconidia at lower drug concentrations. Some arthroconidia appeared intact whilst adjacent arthroconidia were extensively damaged. Arthroconidia had small pores which with time became confluent. Extensive craters were seen invaginating into the structure of the conidia. A pattern of grooves were in evidence on the outer surface of the conidial wall. Portions of the outer wall were detached to expose grooved or ridged deeper layers (Fig 3:9–11). At terbinafine concentrations of 50 and 100 mg/L the arthroconidial wall collapsed. In such assays, conidia were not viable when the stratum corneum sheets were imprinted on glucose–peptone agar. The presence of terbinafine did not prevent adherence of arthroconidia to the stratum corneum surface. Arthroconidia appeared to attach to corneocytes at either the polar or equatorial regions of their cell wall. As this attachment process progressed, the microvilli of the corneocyte appeared to flatten and a depression had formed where arthroconidial attachment had taken place. At 20, 24 and 48h ungerminated arthroconidia were observed.

Untreated germ tubes appeared as cylindrical forms with lateral branching and of variable length. Detailed structures are described in Chapter 2, section 2:5:2. After 16h incubation germ tubes were seen lying on the stratum corneum in close apposition to the corneocytes. Following exposure to terbinafine – saturated corneocytes, arthroconidial germination or extension of germlings was inhibited at 6.2 mg/L. Some germ tubes were dilated and swollen (Fig 3:12) while in others pores appeared along the length of the germ tubes and the lateral branches (Fig 3:13–14). With continued incubation there was a gradual enlargement of the pores in the germ tube cell wall (Fig 3:15) followed by disintegration of the wall and complete collapse of the germ tube at 24h (Fig 3:16). The surface of the germ tube exposed to terbinafine was roughened and there was exfoliation of the outer layer. Penetration of terbinafine exposed corneocytes by the germ tubes was observed at 20h. Morphological changes were more marked at higher drug concentrations and after 24h no true mycelium was observed.

Transmission electron microscopy of untreated arthroconidia revealed regular cytoplasmic membranes surrounded by a smooth bilayered cell wall. Terbinafine treated cells appeared to vary in their response to the drug but in general cell walls had become detached from the cytoplasmic membrane. The outer layer of the cell wall appeared to be the initial target structure and as it became detached, the inner layers became exposed (Fig 3:17). Dilated vacuoles (Fig 3:18) and electron dense particles were seen throughout the arthroconidial cytoplasm (Fig 3:19). Adherence of terbinafine – exposed arthroconidia was not inhibited and close apposition between the arthroconidia and corneocytes was evident. Between the two cell types a fibrillar–floccular material, which was also lining the inside of the crevices could be seen. In the stratum corneum model the corneocytes appeared intact and where adherence was seen the crenated appearance of the corneocyte membrane was absent and a smooth attachment area was evident. Similarly, attachment of germ tubes resulted in the same appearance.

Figure 3:6a

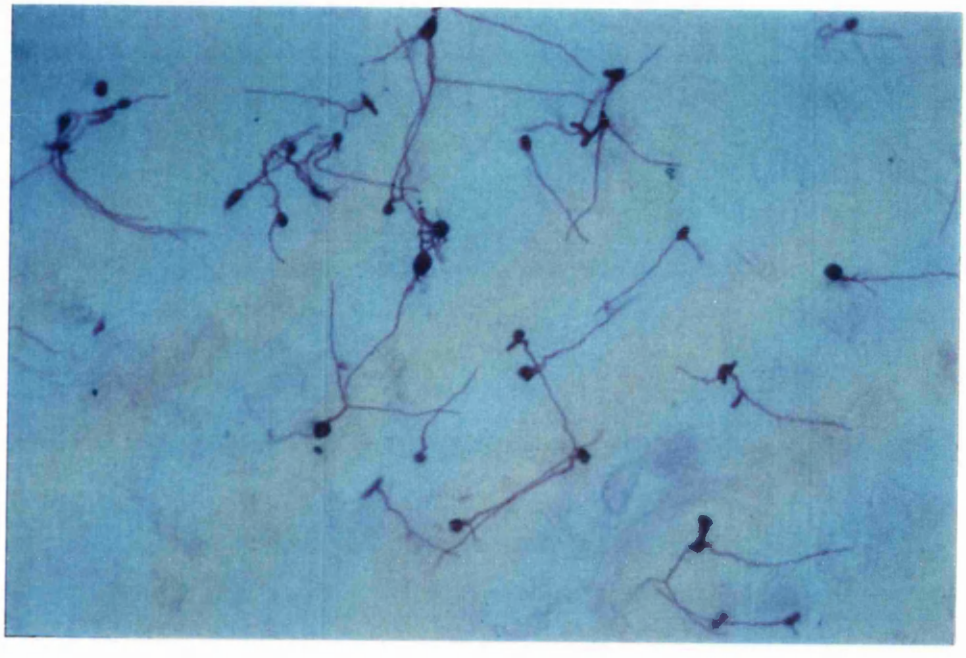


Figure 3:6b

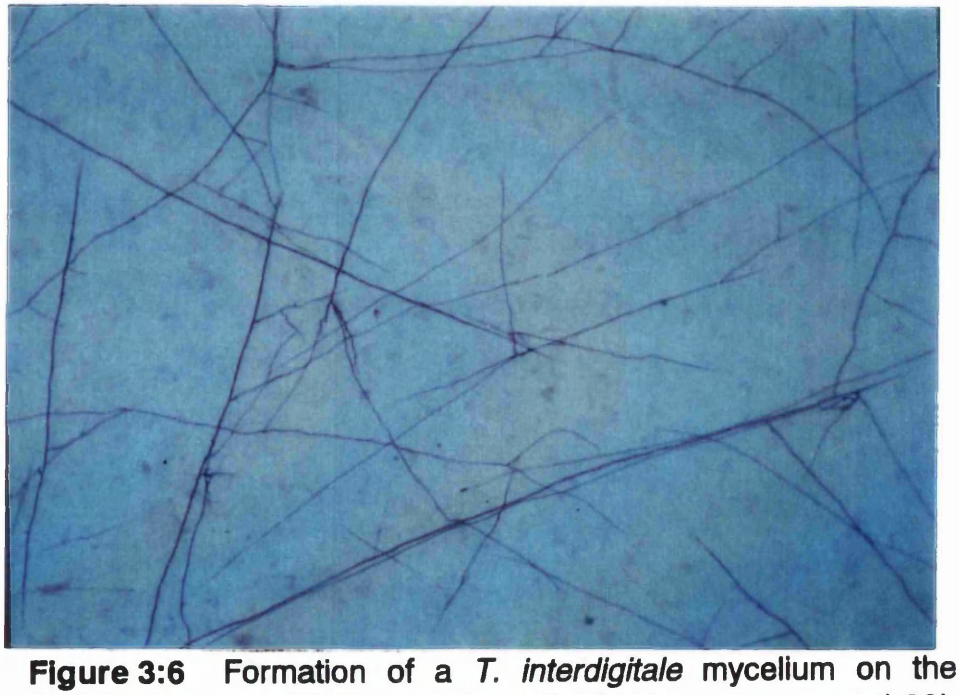


Figure 3:6 Formation of a *T. interdigitale* mycelium on the stratum corneum (a) Germ tubes with side branches around 20h of incubation X 250 (b) Mature mycelium around 40h of incubation X 100. PAS stain

Figure 3:7a

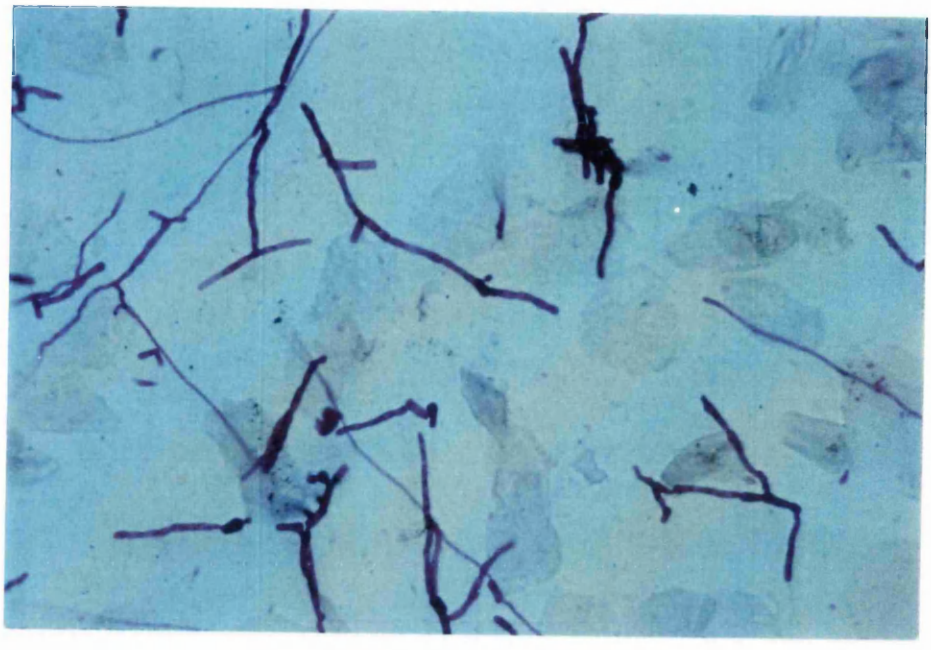


Figure 3:7b

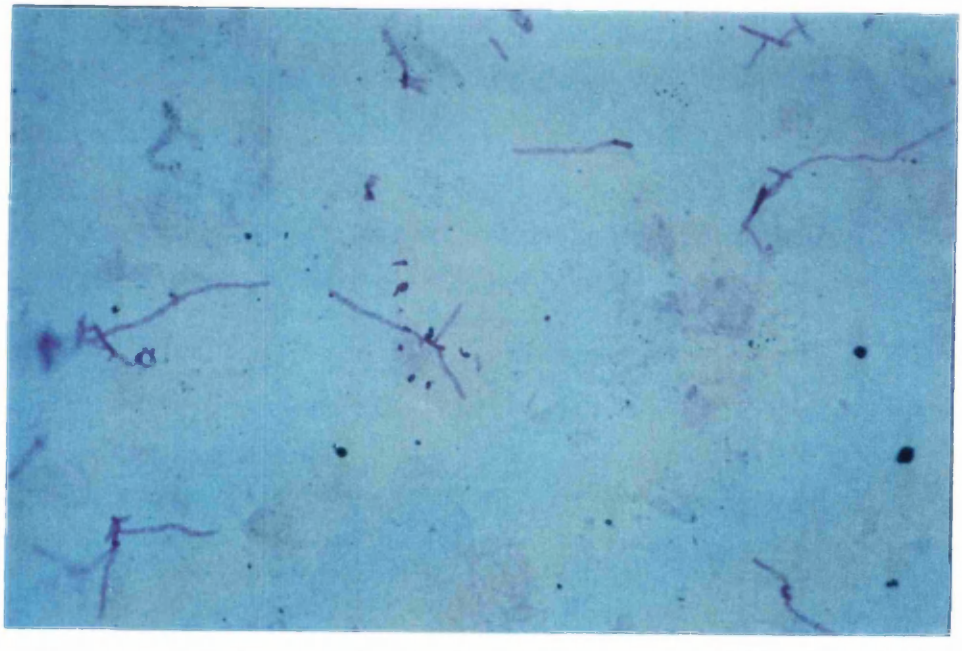


Figure 3:7 (a) Germ tubes of *T. interdigitale* following 20h exposure to 6.2 mg/L terbinafine on the stratum corneum. Note the short and distorted appearance of the germ tubes with evidence of growth inhibition X 400 (b) After 20h exposure to 12.5 mg/L terbinafine. Note the marked inhibitory effect of terbinafine and the lighter appearance of the tubes X 250. PAS stain

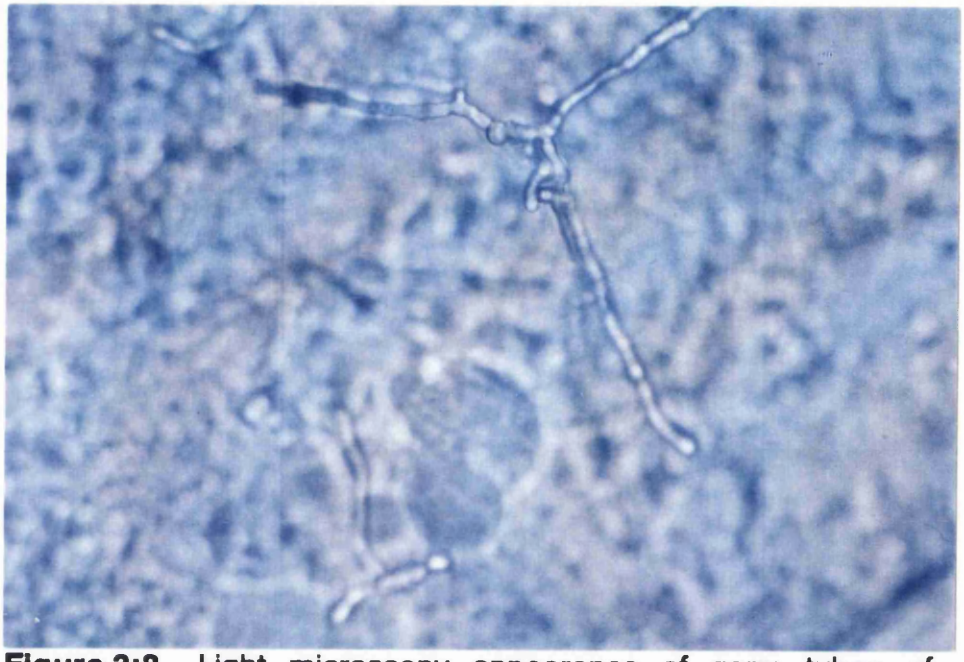


Figure 3:8 Light microscopy appearance of germ tubes of *T. interdigitale* following 20h exposure to 6.2 mg/L terbinafine on the stratum corneum. There is inhibition of growth and the germ tubes appear unhealthy X400.

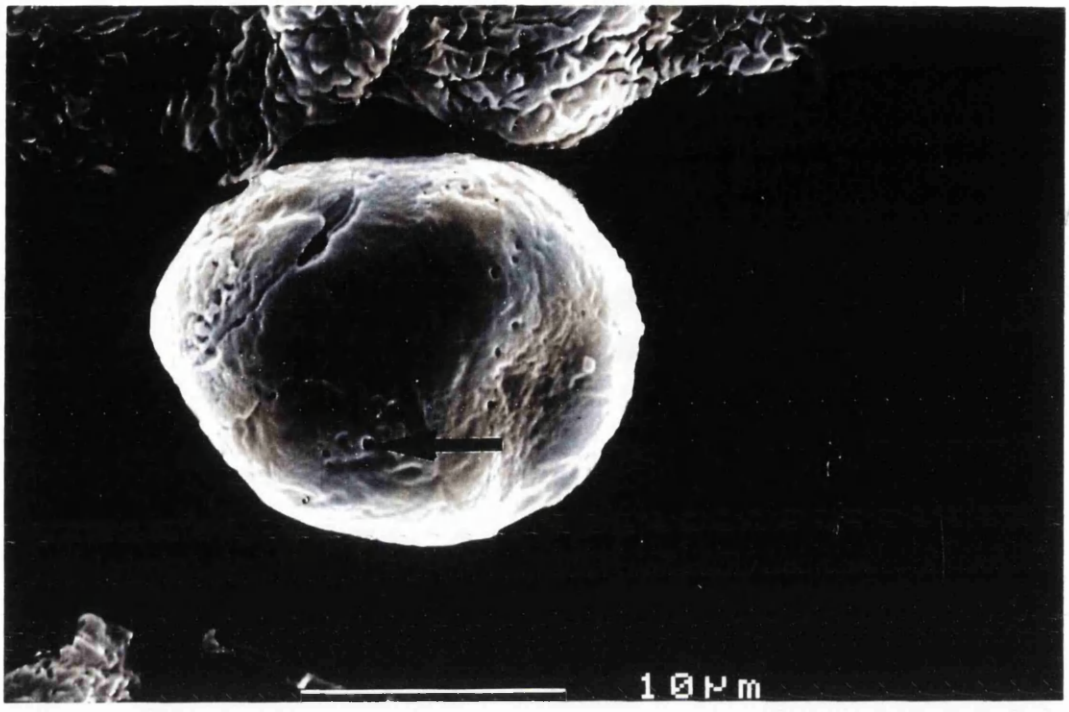


Figure 3:9 Scanning electron micrograph of an arthroconidium of *T. interdigitale* after 16h exposure to 12.5 mg/L terbinafine. Note the small pores in the cell wall (arrow) and the rough exfoliating surface. Adherence to the corneocyte is not prevented X 6000.

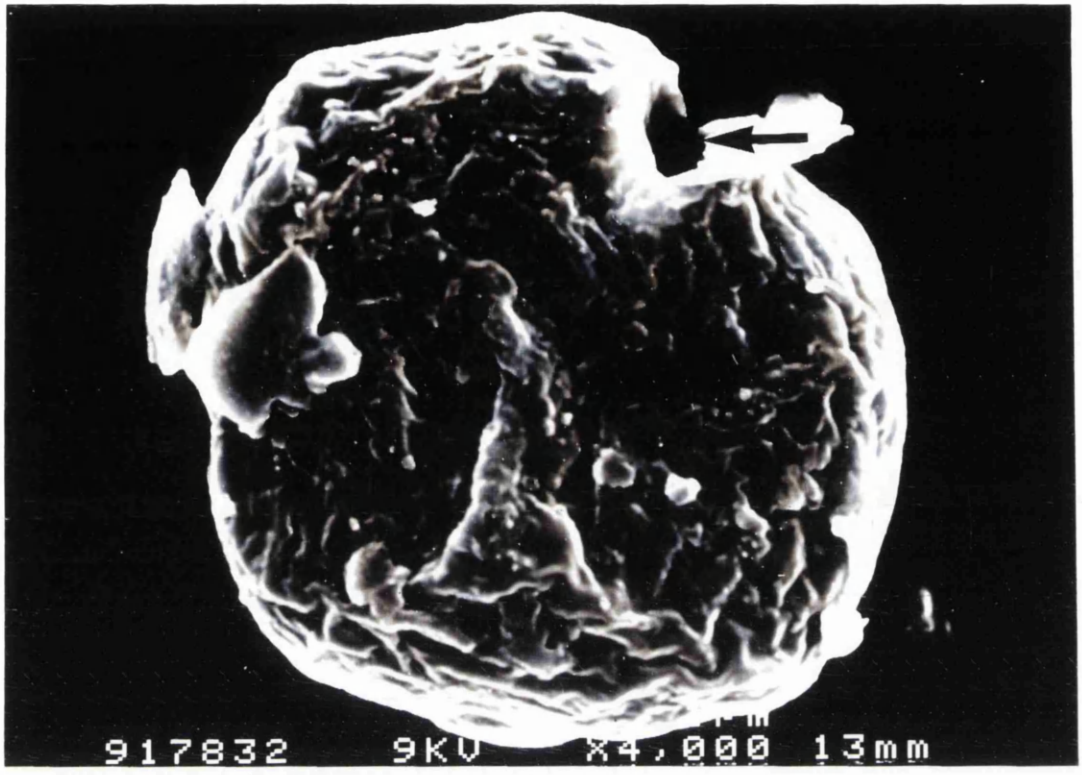


Figure 3:10 Scanning electron micrograph of an arthroconidium of *T. interdigitale* after 16h exposure to 50 mg/L terbinafine in the presence of stratum corneum. Note the pore in the arthroconidium (arrow) and the roughened surface of the cell wall with the outer layers peeling off X 8000.

Figure 3:11a

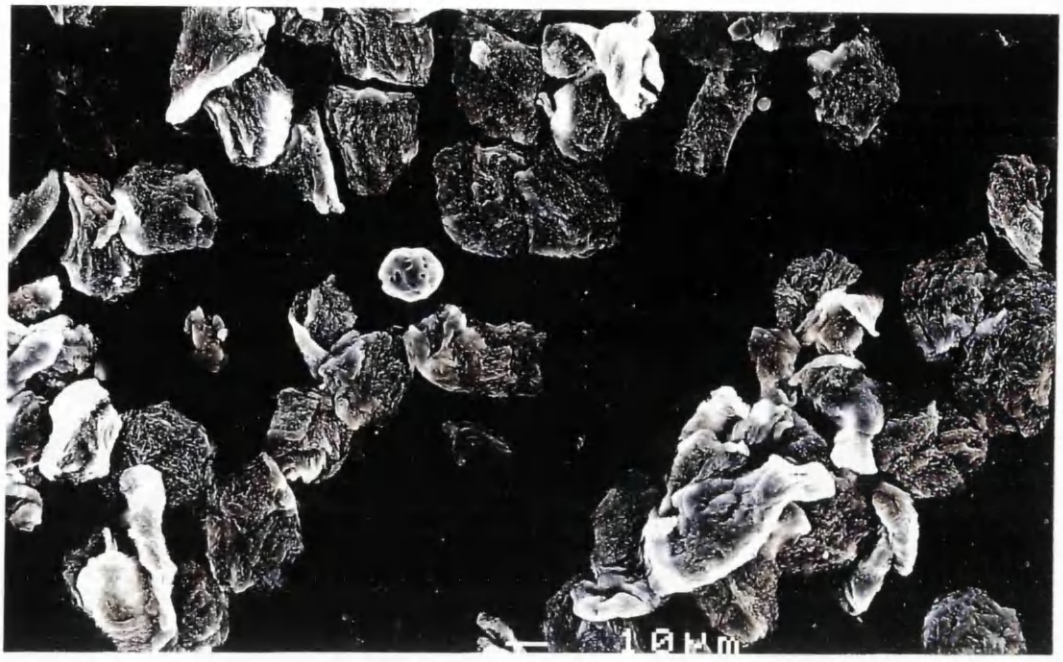


Figure 3:11b

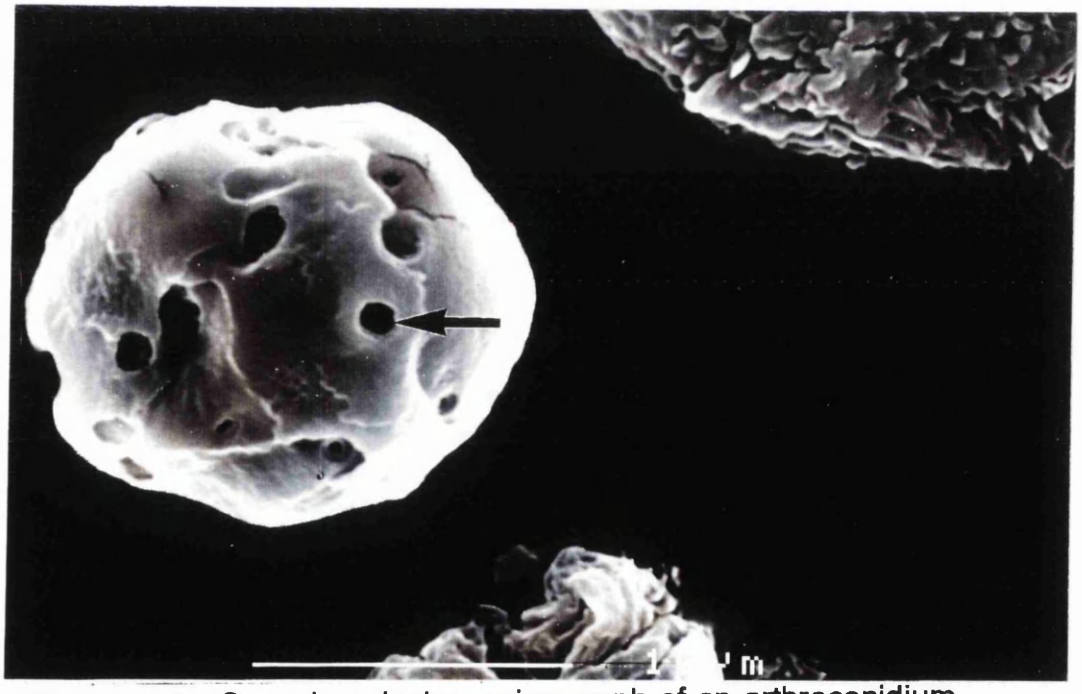


Figure 3:11 Scanning electron micrograph of an arthroconidium of *T. interdigitale* in the presence of stratum corneum after 20h exposure to 100 mg/L terbinafine (a) Note the damage to the arthroconidium X 900 (b) and the deep pores and craters (arrow) on the surface of the arthroconidium X 7400.

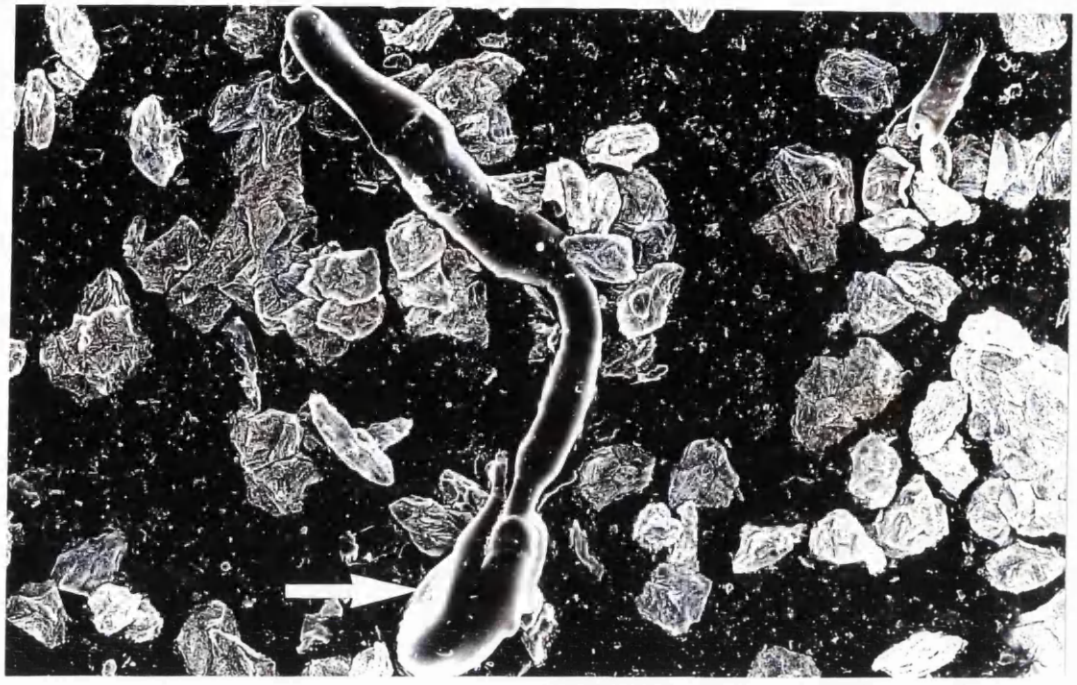


Figure 3:12 Scanning electron micrograph of a germling of *T. interdigitale* after 16h exposure to 6.2 mg/L of terbinafine. Note the swollen filament with the club shaped head (arrow) X 500.

Figure 3:13a

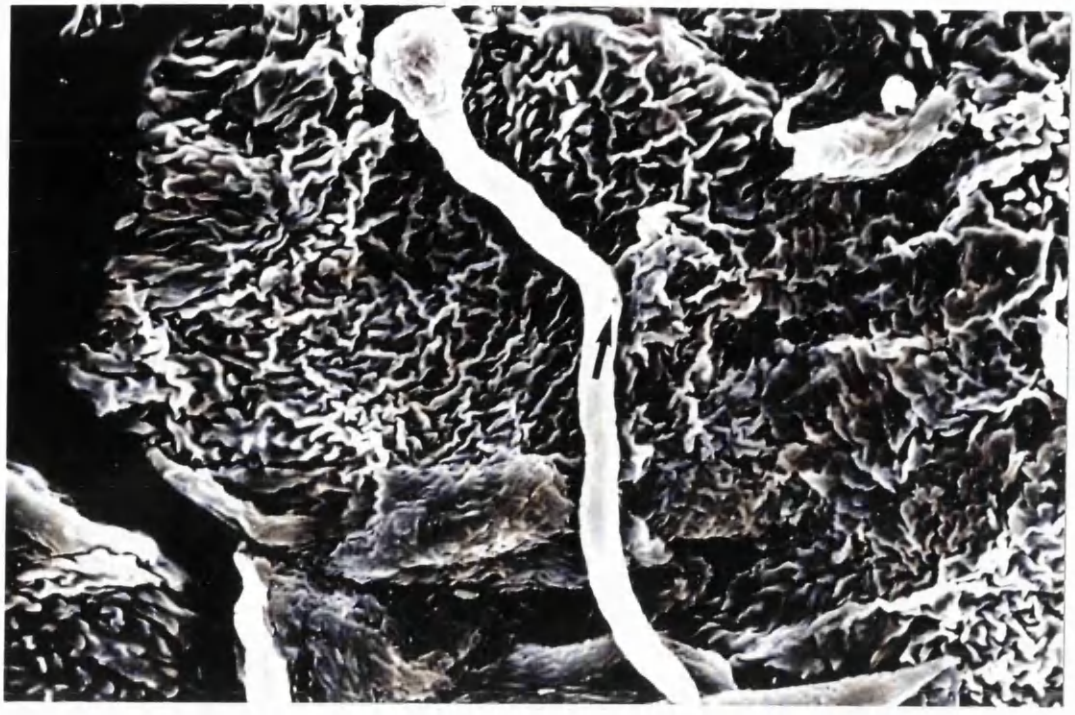


Figure 3:13b

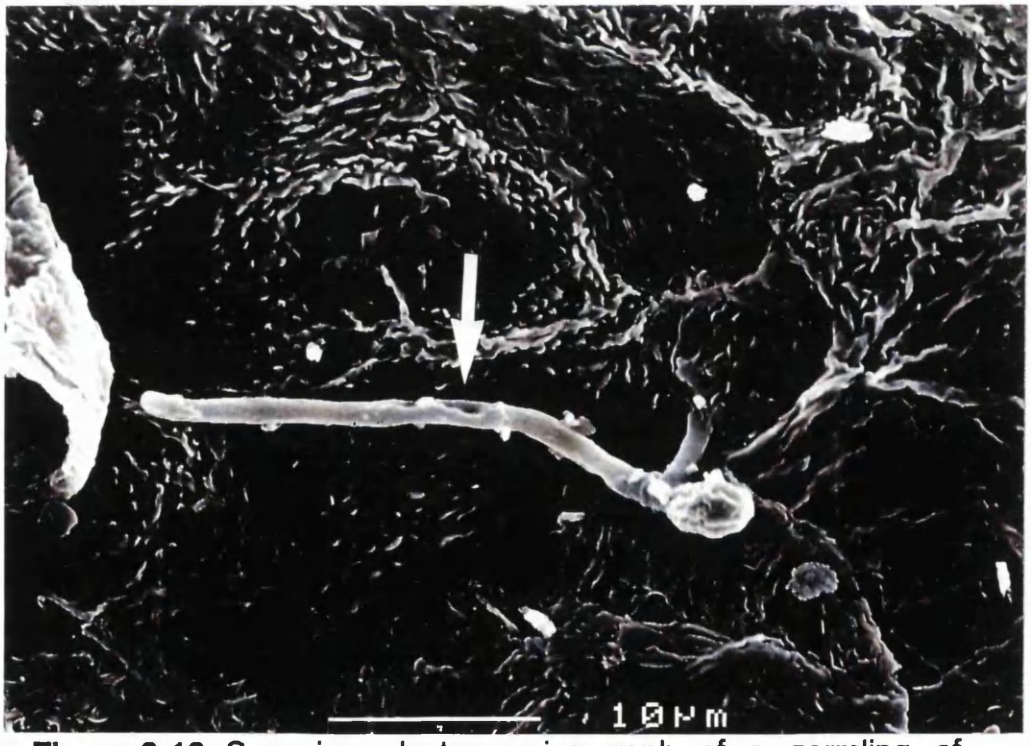


Figure 3:13 Scanning electron micrograph of a germling of *T. interdigitale* (a) after 16h X 7000 (b) After 20h exposure to 12.5 mg/L of terbinafine. Note the pores (arrows) along the length of the filament X 5000.

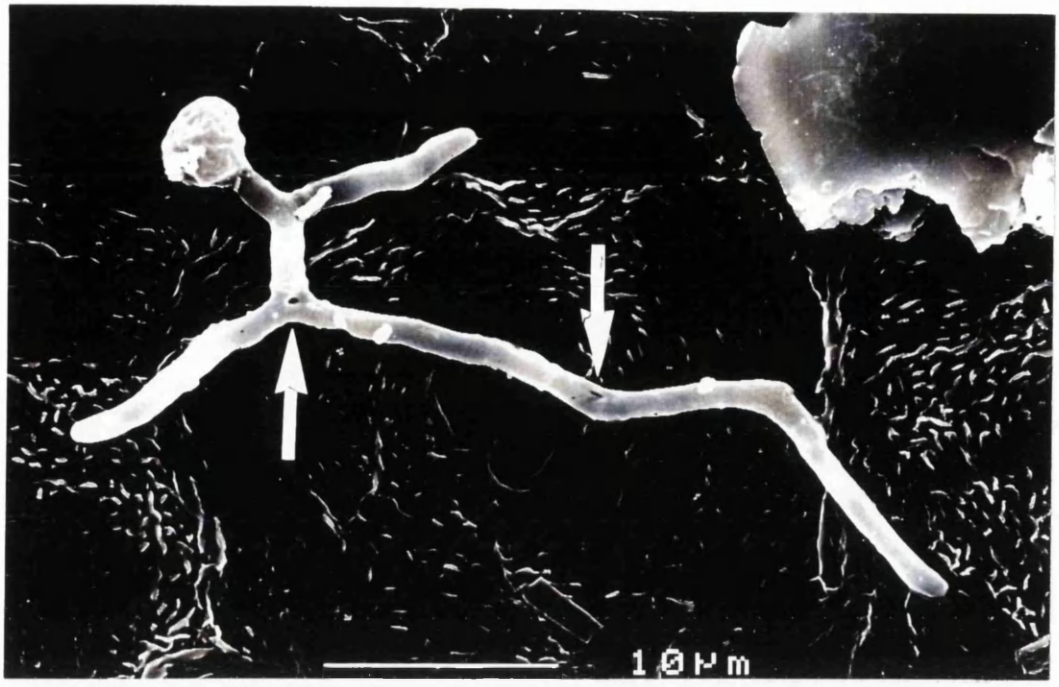


Figure 3:14 Scanning electron micrograph of a germ tube of *T. interdigitale* after 20h exposure to 25 mg/L terbinafine. Note side branching, the roughened surface of the parent arthroconidium and the pores along the length of the filament (arrows) X 5400.

Figure 3:15 Scanning electron micrograph of a germ tube of *T. interdigitale* after 24h exposure to 50 mg/L terbinafine. Note the rough exfoliative surface and the pore in the filament and the side branch (arrows) X 11000.

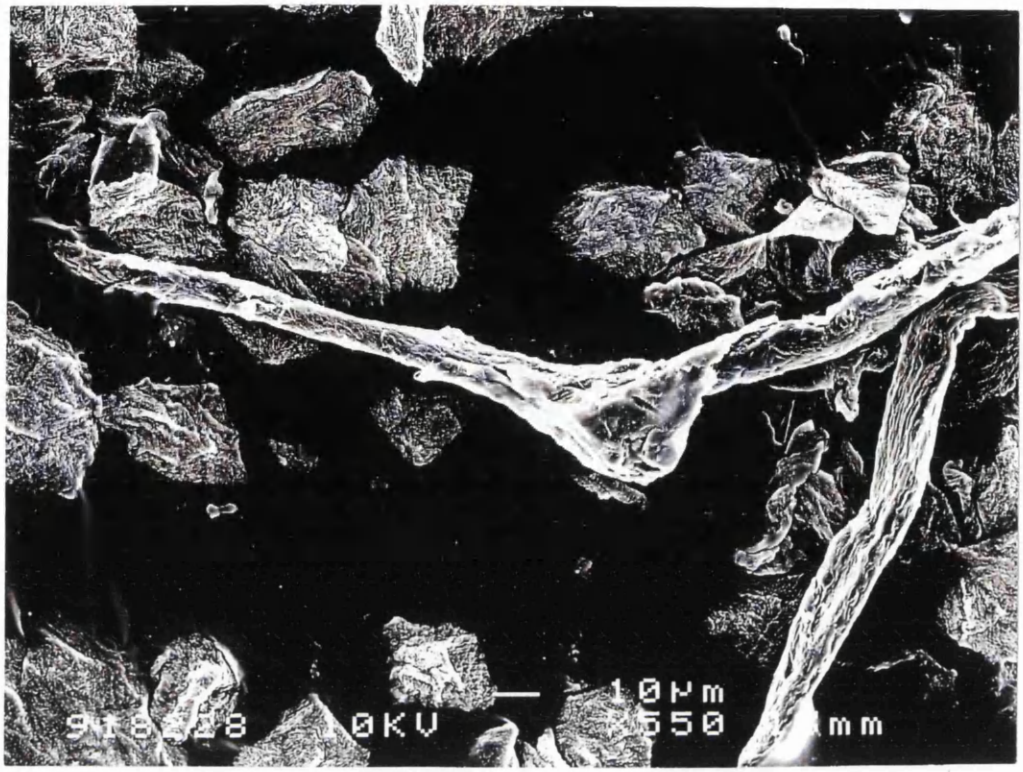


Figure 3:16 Scanning electron micrograph of the remnant of two germ tubes of *T. interdigitale* after 24h exposure to 100 mg/L terbinafine X 1100.

Figure 3:17a

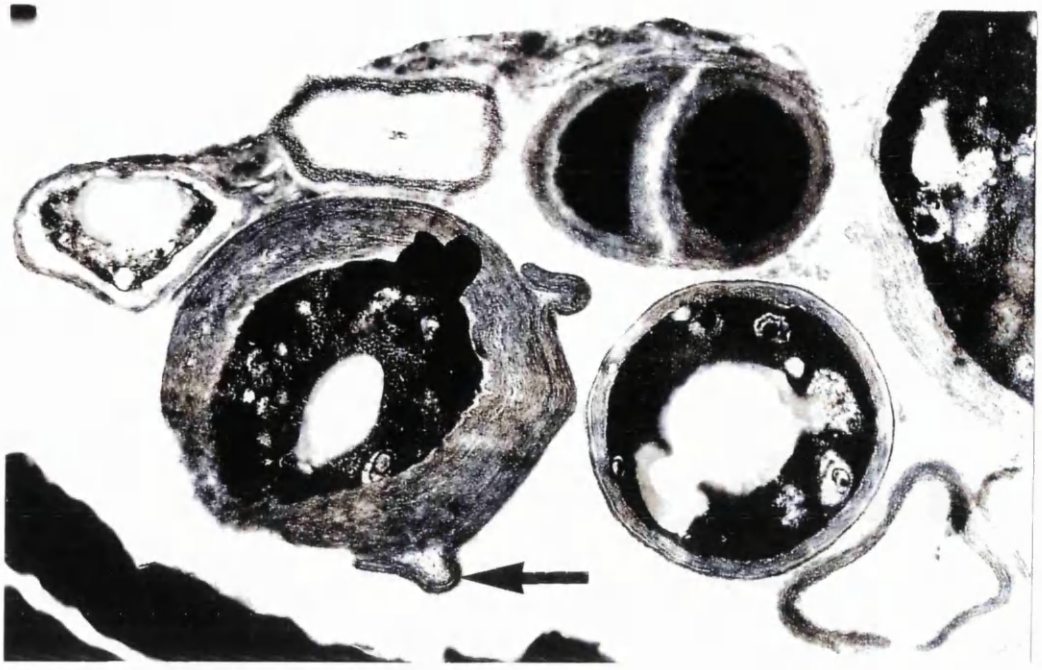


Figure 3:17b

Figure 3:17 Transmission electron micrograph of arthroconidia of *T. interdigitale* after 16h exposure to 50 mg/L terbinafine (a) Note the damage to the outer layer of arthroconidium (arrow) which is peeling off X 15000 (b) close up of the damaged outer layer of the arthroconidium X 75000.

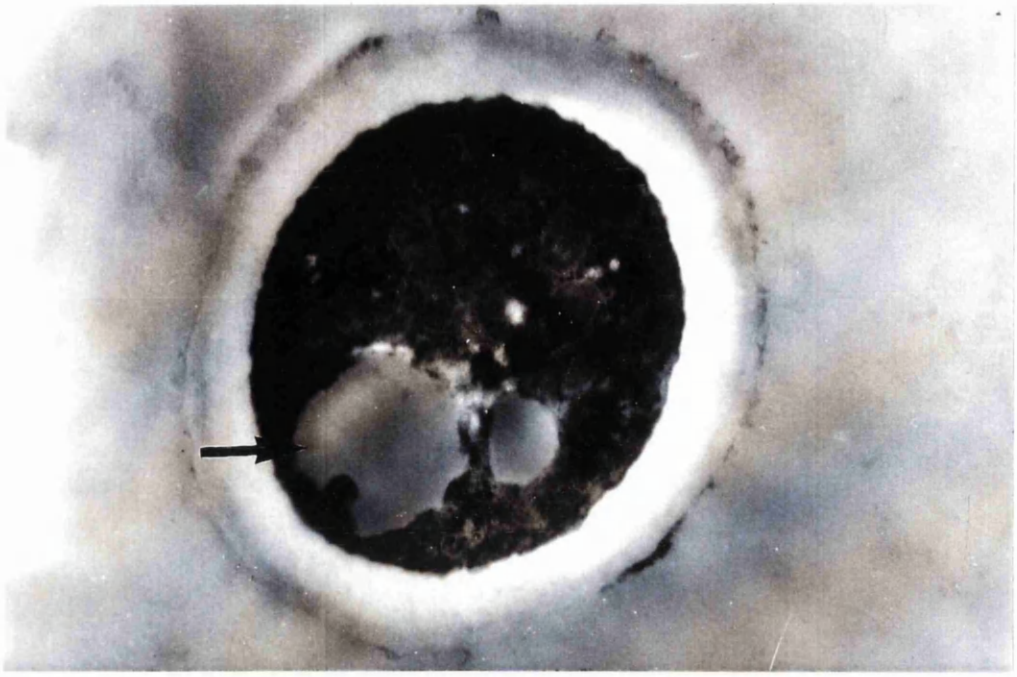


Figure 3:18 Transmission electron micrograph of arthroconidium of *T. interdigitale* after 20h exposure to 6.2 mg/L terbinafine. Note the dilated vacuoles (arrow) in the cytoplasm X 37500.

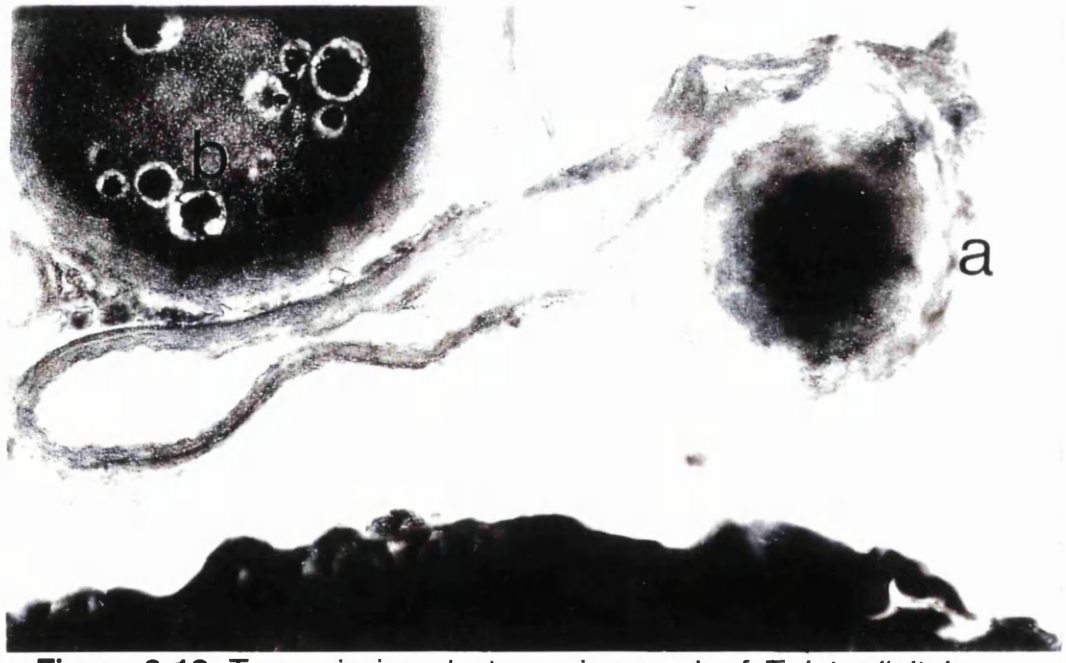


Figure 3:19 Transmission electron micrograph of *T. interdigitale* arthroconidia after 20h exposure to 12.5 mg/L of terbinafine. Note the detached outer surface of arthroconidia "a" and the electron dense inclusions within the cytoplasm of arthroconidium "b" X 18000.

3:6 Discussion

3:6:1 *In vitro* stratum corneum model for the assessment of antifungal agents

The target tissue of dermatophytes is the non-living stratum corneum. Dermatophytes seldomly invade deeper tissues and thus they are not in direct contact with the plasma or any antifungal drug present in this compartment. The various pathways by which antifungal drugs are delivered to the stratum corneum have been described by Artis (1985). Terbinafine is concentrated rapidly in the stratum corneum (up to 9.1 μg/g of tissue) primarily by diffusion from the vascular system through dermis-epidermis and then binds to lipophilic keratinocytes in the stratum corneum (Faergemann *et al.*, 1990). Terbinafine may also be incorporated into the basal keratinocytes and transported to the stratum corneum during normal cell turnover. It has been observed that application of a 1% terbinafine cream attains higher concentration in the stratum corneum than oral administration of terbinafine (Lever *et al.*, 1990). In a trial of patients with tinea pedis and tinea corporis, Villars and Jones (1989) showed that the oral and topical forms of terbinafine are virtually equivalent in their effectiveness. These considerations prompted the use of a stratum corneum model which bears a close resemblance to the *in vivo* environment and focussed upon the choice of the drug concentration range used.

Knight (1973b) introduced the system of using stripped sheets of stratum corneum to assay antifungal activity. He studied the growth of *T. mentagrophytes* microconidia on untreated or normal stratum corneum previously exposed to a antifungal drug. Other studies using stratum corneum have focussed on the bioavailability of antifungal agents and their effect on hyphal development by using cyanoacrylate skin surface stripping (Rurangirwa *et al.*, 1989; Pierard *et al.*, 1991). Gip (1988) used transparent tape to observe the lingering effect of 1% omoconazole cream following a single application.

Aljabre *et al.* (1991) used scraped human corneocytes in an *in vitro* model to study antifungal drugs. The present study is the first where the effect of an antifungal agent on the arthroconidium and germ tubes of a dermatophyte in an *in vitro* stratum corneum model has been studied (Rashid *et al.*, 1993). Others have reported the effect of antifungals on *T. mentagrophytes* hyphae in Sabouraud broth medium (Meingassner *et al.*, 1981; Nishiyama *et al.*, 1991,1992). It is not possible to assay antifungal drug activity on solid media and then compare with the morphological changes induced by direct contact with drug in the stratum corneum as obfuscation of the effect of antifungal agents has been observed in solid media (Hoeprich and Finn, 1972). Again, none of these studies have looked at the changes or damage to arthroconidia.

Terbinafine exerts a potent primary fungicidal action against the dermatophytes (Petranyi *et al.*, 1987). In addition, clinical studies of terbinafine in dermatophytosis have demonstrated rapid antifungal activity with cultures becoming negative soon after commencement of therapy. Berman *et al.* (1992) suggested that the concentration in the skin after a brief course of treatment may be more important than continued application. A recent study has shown that where 1% terbinafine cream was applied to the back over a seven day period the number of applications did not significantly increase the peak concentration in the stratum corneum (Hill *et al.*, 1992). However, there was an increase in the total amount of terbinafine found in the stratum corneum. Because of these clinical studies and the primarily marked fungicidal action of terbinafine and the possibility of short therapy for dermatophytosis, short contact times and short period of assessment were used in this study. Based on the findings in Chapter 2, whereby active fungal growth was taking place on the stratum corneum at 20h and the observation that the fungicidal effect of terbinafine appeared following 20h incubation, it was decided to measure the inhibitory effect of terbinafine on the growth of *T. interdigitale* at this time.

3:6:2 Effect of terbinafine on the germination of arthroconidia on different body surfaces

Although arthroconidia are the natural pathogenic elements of dermatophytes and the most relevant morphological form of these fungi to use in antifungal susceptibility tests, they have rarely been used in studies with antifungal drugs. Aljabre *et al.* (1991) used pure arthroconidia in corneocyte suspension to study the effect of clotrimazole and griseofulvin on germination but did not assess the morphological changes in arthroconidia. Other studies (Wright *et al.*, 1983; Scott *et al.*, 1984) have used arthroconidia in sensitivity testing of antifungal agents but these tests were conducted in laboratory media. In the present study the effect of terbinafine on dormant arthroconidia was observed. On inoculation onto sheets of stratum corneum, arthroconidia started to germinate in the controls while arthroconidia inoculated on terbinafine exposed sheets of stratum corneum showed varying degrees of inhibition of germination on all body surfaces examined. Arthroconidia appeared to be more susceptible to drug induced damage than germlings. More morphological changes were seen in the arthroconidia at lower drug concentrations than in the germ tubes. Varying degrees of damage to the arthroconidia was observed from pores to craters on the surface and totally destroyed conidia were seen. Damaged arthroconidia and germlings were non viable.

Germination of *T. mentagrophytes* arthroconidia is the first stage in the development of tissue invasive hyphae and the formation of vegetative mycelium. Measuring the effect of terbinafine during this phase of active fungal growth may reflect antifungal activity *in vivo* more closely than conventional MIC tests (Scott *et al.*, 1985). In the present study high concentrations of terbinafine have been used whereby the drug has been incorporated in the stratum corneum. Scott *et al.* (1984) indicated that higher concentrations of antifungal drugs are required to inhibit germination of arthroconidia and also showed that dormant arthroconidia were more resistant to antifungal agents.

The use of stratum corneum to assay antifungal drugs helps in breaking the dormancy or resistance of the arthroconidia. Aljabre *et al.* (1991) found that clotrimazole and griseofulvin inhibited germination of activated arthroconidia in corneocyte suspensions.

The inhibitory effect of terbinafine on germination and the morphological changes observed in the arthroconidia in the presence of terbinafine could be due to a number of factors. Firstly, due to direct damage to the arthroconidial wall as described by Nishiyama *et al.* (1991). Secondly, dormant arthroconidia are usually resistant to commonly used agents such as clotrimazole, griseofulvin, miconazole nitrate and nystatin (Hashimoto and Blumental, 1978). At the time of germination, resistance of the arthroconidia to the antifungals is completely lost. Subsequently, drug penetration to target sites is hindered by the arthroconidial wall due to its impervious nature (Hashimoto, 1991). As germination proceeds, increased permeability of the arthroconidial wall renders the conidium more susceptible (Scott *et al.*, 1984). Thirdly, damage to the non-fibrillar chitinase resistant layer which surrounds the arthroconidia against antifungal agents (Pollack *et al.*, 1983). Finally, the electron dense bodies seen in the present study (Rashid *et al.*, 1993) are very similar to those seen when *T. mentagrophytes* was exposed to naftifine (Meingassner *et al.*, 1981). They demonstrated the lipid nature of these bodies and speculated that they were formed as a result of squalene accumulation and either transported centripedally into vacuoles or alternatively to the cell surface by exocytosis. It may be that the aggregation of these vesicles on both sides of the plasma membrane resulted in structural changes seen in the arthroconidia and germ tubes induced by terbinafine.

Electron microscopy studies showed that adherence of arthroconidia to corneocytes which had previously been exposed to terbinafine was not inhibited. These findings suggested that either the amount of drug released from the corneocytes was not enough to inhibit the process or alternatively that

terbinafine has no effect on the adherence process of dermatophytes to corneocytes.

3:6:3 Effect of terbinafine on germ tube extension on different body surfaces

The findings in this study show that terbinafine had a variable inhibitory effect on germ tube extension on different body surfaces. At lower concentrations, dilated germ tubes with swollen ends were seen. At higher concentrations pores were observed in the germ tubes and their side branches and collapsed germ tubes were seen lying on the stratum corneum. The morphological changes observed in the germ tubes of *T. mentagrophytes* caused by terbinafine were associated with growth inhibition and killing activity which was drug concentration and time dependent.

Scott *et al.* (1985) looked at the effects of ketoconazole and miconazole on hyphal development in *T. mentagrophytes* and found that germ tube extension bears a closer relationship to MIC's for arthroconidia than does percent germination. Other studies have reported the effect of antifungals on *T. mentagrophytes* hyphae in Sabouraud broth medium (Meingassner *et al.*, 1981; Nishiyama *et al.*, 1991). Meingassner *et al.* (1981) used low concentrations (0.1–0.5 μg/ml) of naftifine and found swelling of hyphal tips as well as concentration–dependent formation of homogeneous electron dense bodies in the cytoplasm. Nishiyama *et al.* (1991) showed that terbinafine inhibited hyphal growth at concentrations above 0.2 ng/ml and also described swelling of hyphae and electron dense granular structures in the cytoplasm. At higher concentrations of terbinafine the hyphae were destroyed or collapsed. They speculated that the electron dense structures were accumulated squalene and that terbinafine may have a direct damaging effect on the cell membrane. Some of the findings in this study are similar to those described above. Electron dense structures were seen in the cytoplasm of arthroconidia and the

peeling of the arthroconidial cell wall as seen in Fig 3:19 could be due to the direct damaging effect of the drug.

Terbinafine's fungicidal action is very marked. The drugs antifungal activity is based on the inhibition of the fungal enzyme squalene epoxidase. This enzyme is important in the biosynthesis of ergosterol, an essential constituent of the fungal cell membrane. Inhibition of squalene epoxidase leads to a decrease in membrane ergosterol and accumulation of intracellular squalene, both of which are thought to contribute to the fungicidal action of terbinafine (Ryder and Mieth, 1992). Although ergosterol synthesis is only partially inhibited (approximately 20%) cell growth is completely arrested. These findings suggest that the fungicidal effect of terbinafine may be related to the accumulation of squalene. Fungicidal action shows a relatively slow onset, which follows the time course of squalene accumulation. It is assumed that sensitivity against high squalene levels within the cells gives rise to fungicidal effects. The precise action of intracellular squalene is not clear, but it may increase membrane fluidity leading to disruption of enzyme function and cell structure.

In the present study a slow onset of the fungicidal effect of terbinafine was observed. It was marked and rapid following 20h incubation and maximal around 24h. Collapsed hyphae were seen lying on the stratum corneum following exposure to terbinafine, at 24h. The time lag phase following exposure to terbinafine and the onset of the fungicidal effect could be explained by squalene accumulation within the fungus. Lack of readings following 3h exposure on the leg and sole was because of the fungicidal effect setting in early and, therefore, it was not possible to quantify readings clearly. At lower drug concentrations lateral branching from germ tubes occurred. Furthermore, hyphal tips were observed to penetrate through and between corneocytes which had previously been exposed to terbinafine, suggesting that the amount of drug released from the corneocytes was not inhibiting the invasion process.

Terbinafine is highly lipophilic and is distributed preferentially to the skin and sebum, as well as fatty tissues (Ryder and Mieth, 1992). From these tissues it is released slowly. The elimination half life of terbinafine from the stratum corneum and sebum is 3–5 days and therefore concentrations above the MIC for most dermatophytes may be present up to 3 weeks after cessation of oral therapy (Faergemann *et al.*, 1990). This distribution pattern of terbinafine may be partially responsible for its high efficacy in chronic diseases of thickened skin or nails. Furthermore regional variation in the stratum corneum from different body surfaces has been described (Holbrook and Odland, 1974). Similarly, corneocytes are known to vary in size, shape and surface area with the region of the body, age, sex and season (Plewig, and Marples 1970). The permeability of the stratum corneum varies with different body regions. These variations could possibly explain the variable effect or release of terbinafine from the corneocytes and the effect on the dermatophyte growing on the stratum corneum. Differences in the distribution and avidity of the stratum corneum from one part of the body to another for terbinafine may be expected to affect the activity of this antifungal agent. The existence of such differences in the distribution of griseofulvin in the stratum corneum has been described (Epstein *et al.*, 1972). It was clear that longer exposure of the corneocytes to terbinafine was more effective in inhibiting germ tube elongation, possibly because more drug is absorbed by the corneocytes and is available to be released.

3:7 Conclusion

The results of this study showed that terbinafine partially inhibited germination of *T. interdigitale* arthroconidia but germ tube elongation was inhibited indicating that the drug has a delayed effect on cellular differentiation. By reducing the rate of germ tube extension terbinafine could abrogate the pathogenic ability of the dermatophytes. Contrary to previous reports about the

resistance of dormant arthroconidia to antifungal drugs this study suggests that the arthroconidial outer and inner layers are susceptible to terbinafine with subsequent fungicidal alternations to the cytosol and intracellular organelles.

The immediate clinical implications of this study are that prophylaxis with terbinafine for ringworm in endemic areas may be highly effective since arthroconidia coming into contact with the stratum corneum will be damaged and morphological transition to the hyphal form will be inhibited. Also, a single oral dose or topical application of terbinafine may result in accumulation of the agent in the stratum corneum at a level higher than the minimum mycelial growth inhibitory level. Clinical experience with terbinafine and the present work demonstrate the potential of short-term treatment courses with terbinafine for dermatophytosis.

The stratum corneum model described in this study can be used for the *in vitro* assessment of antifungal drugs and to observe their effect on the morphology and ultrastructure of the dermatophytes growing on it. Also it can be adopted to test for the bioavailability, penetration and stability of proprietary antifungal preparations within the stratum corneum. It could have a wider application in clinical trials for testing the activity of drugs against human fungal infections in case of therapeutic failure. The advantage of this method is that it simulates the physiological environment of dermatophyte fungi.

Chapter 4

Colonization and invasion of nails by dermatophyte fungi

4:1 Summary

A new *in vitro* model for the study of nail invasion by dermatophyte fungi was developed. Two dermatophytes, *T. mentagrophytes* and *T. interdigitale* and finger and toe nails were used. Arthroconidia were inoculated on the ventral surface of the nails. 6h after inoculation adherence and germination of arthroconidia was observed. By 16h, small germ tubes with side branches were evident. Around 24h, micro-colonies were observed. At 48h a mycelium had formed and around 72h most of the nail fragment was covered with fungal growth. Nail penetration was seen from the ventral surface through the intercellular spaces and with longer incubations all three layers were invaded with arthroconidia growing through channels. Nail invasion was seen in the absence of added nutrients. Dermatophyte fungi appeared to invade the nail by a combination of mechanical and chemical factors.

4:2 Introduction

Nail infections are known by two names. Onychomycosis literally means 'fungal infection of the nail' while tinea unguium is specifically confined to dermatophyte nail infections. The most common type of nail infection (fungal) is the distal subungual tinea unguium. The keratin of the hyponychium becomes infected and the infection progresses then involves the nail bed and subsequently the nail plate (Zaias, 1972a). Most dermatophyte species invade the ventral layer first as it is attached to the nail bed (Achten and Simonart, 1964). Occasionally the intermediate layer is involved in the infection process. The dorsal layer of the nail plate is rarely infected except in white superficial onychomycosis, which occurs almost exclusively in toe nails (Zaias, 1972b).

The dermatophytes commonly associated with tinea unguium are *T. rubrum* and *T. mentagrophytes var interdigitale*. The latter is normally seen only in toe nail infections. Some fungi can invade the nail plate more easily

than others. Zaias (1972a) found that *T. mentagrophytes* was a more active destroyer of nail tissue than *T. rubrum.* However, the mechanism of such nail destruction remains unclear. It has been speculated that a mechanical (Raubitschek and Maoz, 1957) or enzymatic process (*Yu et al.*, 1972) may be involved. For example, *T. mentagrophytes* is capable of producing proteolytic enzymes, keratinases (*Yu et al.*, 1971).

Very few *in vivo* and *in vitro* attempts have been made to induce nail penetration by dermatophytes. *In vivo* attempts to induce tinea unguium have had varied success. Vilanova *et al.* (1956) attempted to induce experimental nail infections in normal finger nails. 216 inoculations were made in various nails by different methods and with different fungi in nearly all subjects. Only 52 showed evidence of lesions which were dependent on the site of inoculation. *In vitro* studies looking at the process of nail invasion by dermatophytes are few. Raubitschek and Maoz (1957) added nail clippings to a flask of simple medium under conditions of continuous shaking to simulate the parasitic life cycle of dermatophytes. They found evidence of nail penetration based on histological sections stained with PAS stain. Achten and Simonart (1964) placed nail clippings in medium and inoculated these with mycelial suspensions. There was evidence of fungal invasion in the nails as observed on light microscopy. Rosselet and Frenk (1990) attempted nail invasion by adding different media to enhance nail penetration. They found that nutritional factors can alter the rate of nail invasion by dermatophytes.

A complete evaluation of onychomycosis must include study of the clinical aspects, light microscopy, electron microscopy and identification of the invading organism on suitable culture media. Previous studies have attempted to explain how the nail plate is involved clinically and the possible invasion of the nail from the lateral nail grooves and from the hyponychium (Zaias, 1972a). Such studies have studied the structure of the nail in relation to the nail bed and the structural organisation of these two structures (English, 1976). Many studies on fungal diseases of the nails and histological investigations have been

carried out (Sagher 1948; Jillson and Piper 1955; Suarez *et al.*, 1991). However, conventional light microscopy observations gives only limited information about the spatial arrangement of fungal elements within the nail and about the fine structural details of penetration into the nail plate. Electron microscopy studies are rare. For example, Meyer *et al.* (1981) looked at avulsed mycotic nails under scanning electron microscopy and found that it provided more detailed information about the interaction between the dermatophyte and the nail.

In the nails, dermatophytes exist as arthroconidia. To simulate the process of nail invasion *in vitro* it is important to expose the nails to the actual pathogenic elements of the dermatophytes; that is, the arthroconidia. Nail invasion by dermatophyte arthroconidia has not been carried out, to date, neither has the process of nail colonization and invasion been studied at an ultrastructural level in detail with scanning and transmission electron microscopy. This is the first thorough investigation concerning the process of nail invasion by dermatophyte fungi in the absence of nutrients.

4:3 Aim of the Study

This study was undertaken to investigate the ability of *T. mentagrophytes* arthroconidia to adhere and morphologically transform on nails and to observe the process of nail colonization and invasion by germlings of *T. mentagrophytes* in the absence of added nutrients. This study would enable an examination of the possible routes of nail penetration and to visualise the spatial arrangement of the fungus in the nails, further adding to our knowledge of the growth of dermatophytes on keratinous substrates.

4:4 Materials and methods

4:4:1 Organisms and stock culture

One strain of *T. mentagrophytes* (Strain 126 and one strain of *T. interdigitale* (Chapter 2, section 2:4:1) were used. The strain of *T. mentagrophytes* was preserved on silica gel at 4°C. Initially it was recovered on GPA at 28°C from silica gel. Subcultures were maintained on GPA and incubated at 28°C for 10 days in order to obtain an optimal yield of microconidia.

4:4:2 Production and preparation of arthroconidia

Arthroconidia were produced and prepared to a concentration of 5 x 10^4 arthroconidia per ml as described in Chapter 2, sections 2:4:3 and 2:4:4. Viability of singlet arthroconidia was determined as described in Chapter 2, section 2:4:4.

4:4:3 Preparation of nail fragments

Distal fragments of normal human finger and toe nails were used for this experiment. The nails were collected from a healthy male volunteer who was not receiving antifungal therapy. No antiseptic swabbing of the nails was carried out. The nails used in the experiment were examined mycologically and histologically in order to exclude contamination by fungi other than that inoculated on it.

4:4:4 Inoculation of nails

Nail fragments from finger and toe nails were prepared and then mounted on glass slides with the ventral surface uppermost. The glass slides were supported on bent glass rods in a sterile glass petri dish containing 5 ml distilled water. 50 ml of the arthroconidial suspension was inoculated on the ventrial surface of the nail. The nails were incubated for varying lengths of time; 4, 6, 16, 24, 48 and 72 h, 7, 14, 21, and 30 days. For control purposes nail fragments without arthroconidia were incubated at 28°C for various lengths of time.

4:4:5 Assessment of nail invasion

Two methods were used to assess nail invasion. For light microscopy, nails were prepared as in section 4:4:4. Nail scrapings were examined by placing them on a glass microscope slide and then 20% (w/v) potassium hydroxide was added and a coverslip applied. For PAS and H and E stains nails were fixed in 4%(v/v) formaldehyde after growth had proceeded for the desired time. Samples were dehydrated in increasing concentrations of ethanol (25–100%) and embedded in paraffin. Sections (8 – 10 μm thick) were stained with PAS for examination.

For electron microscopy, nail fragments were prepared as in section 4:4:4 and after growth had proceeded for the desired time they were fixed and processed for scanning and transmission electron microscopy as described in Chapter 2, section 2:4:9.

4:5 Results

4:5:1 Normal nail structure

Light microscopy of the nail revealed flattened squamous cells, closely apposed to each other, arranged in lamellae (Fig 4:1). The three dimensional morphology of normal nails on scanning electron microscopy showed lamellar cells of the dorsal nail plate present as a comparatively smooth surface which overlapped each other and presented a tile-like appearance. On lateral view of the nail plate, the three layers, i.e. dorsal, intermediate and rough ventral layers, could be seen. At higher magnification the lateral edge exhibited parallel rough edges. The ventral surface which was in contact with the hyponychium showed a highly irregular topography due to irregular surface structure of this part of the nail plate (Fig 4:2). There were regular, parallel grooves and ridges. The parallel ridges consisted as seen at higher magnifications of parallel fibres which stuck close together. On transmission electron microscopy the fully mature cells of the nail plate showed marked tortuosity of their membranes. The cells presented numerous digitations which merge with those of neighbouring cells (Fig 4:3). The cell cytoplasm was filled with tightly packed keratin filaments combined in bundles with an intervening, relatively electron-dense amorphous matrix. These bundles lay in no precise direction. No nuclear residues or those of cytoplasmic organelles were seen in the sections. The intercellular links between cells were tight.

4:5:2 Germination of arthroconidia and growth of germlings of *T. mentagrophytes* on nail fragments

On gross examination, growth was first visible on finger nail fragments around 4 days and by 6 days on the toe nail fragments. It was initially slow though gradually increased with further incubation. By 7 days the nail

fragments were totally covered by a white fungal mycelium (Fig 4:4). Growth of the mycelium on the nail fragments depended on the size of the fragment and time of incubation. Growth was quicker and more extensive on the finger nails and on smaller fragments and increased with time of incubation. By 14 days nail fragments were totally submerged in aerial mycelia, were not visible but were friable. Around 21 days of incubation finger nail fragments had started to disappear. By 4 weeks of incubation nail fragments had disappeared and only the mycelial mass was left on the glass slide. Toe nails were still present at 4 weeks, but were friable.

Scrapings from nails digested with potassium hydroxide showed hyphae growing on the surface of the nail which gradually extended across and covering most of the nail surface by 72h. By 4 days arthroconidia were seen forming in the hyphae growing on the nail surface. By 7 days all of the nail fragment was covered with growth, with hardly any nail substance visible.

PAS and H and E stained sections showed that the initial penetration of the nail plate took place from the ventral surface as hyphae between cells. Transverse sections showed that the fungi grew between the laminations. With longer incubation the whole nail plate was invaded, with fungi seen in the dorsal, intermediate and ventral layers around 2 weeks of incubation at 28°C. Chains of spores were seen between the nail lamellae, with slits seen (Fig 4:5). Fungi grew most of all in the intercellular space of the nail.

On scanning electron microscopy, at 6h following incubation, arthroconidia were observed to adhere to corneocytes on the ventral surface singly, in pairs or clusters. Arthroconidia were seen attached to the tips of nail corneocytes (Fig 4:6). In other areas a depression was formed where the arthroconidia were adherent. Some arthroconidia were seen lying deep in the crevices on the ventral surface of the nail. Arthroconidia were spherical or oval with a smooth surface, approximately 8–10 μm in size. First evidence of arthroconidial germination was seen at 6h. Germinated and ungerminated

arthroconidia of various shapes and sizes were observed adhering to the nail surface.

By 16h small germ tubes were seen on the ventral surface with side branches appearing and around 24h small micro-colonies had formed on the ventral surface with tips of the germ tubes penetrating the crevices (Fig 4:7). By 48h a mature mycelium was present with hyphae extending peripherally and along the lateral edges of the nail to reach the dorsal surface and penetrated through the parallel crevices on the lateral edge of the nail. The hyphae appeared as cylindrical forms which were of variable length. Their diameter was about 1μm. Side branches were seen arising from and penetrating the corneocytes. Penetration of the nail on the dorsal surface between the corneocytes and through corneocytes was seen. Hyphae were observed to insinuate between or under corneocytes (Fig 4:8). On the ventral surface the gaps, slits and ridges provided an excellent channel for the hyphae to penetrate the nail plate. Similar observations were seen on the toe nails. Bacteria were seen adhering to arthroconidia on their surface and along the length of the hyphae.

Transmission electron microscopy showed arthroconidia adhering to the ventral surface. Where adherence was seen to take place a depression was formed. Adherence between the arthroconidial wall and the tip of the nail corneocytes was very close with a fibrillar-flocullar material in the intercellular space (Fig 4:9). Hyphae and arthroconidia were seen penetrating through the crevices at the lateral edge of the nail (Fig 4:10). By two weeks more extensive invasion of the nail plate was seen, with dorsal, medial and ventral layers invaded. Arthroconidia were present in chains growing through the nail in well formed channels which were lying parallel to each other. By two weeks the nail structure was disorganised, with the keratin bundles lying separately in a disorganised way (Fig 4:11). Penetration of the nail tissue was seen to be occuring through the intercellular spaces. Separation of the cells at the intercellular junctions was observed (Fig 4:12). On longitudinal section

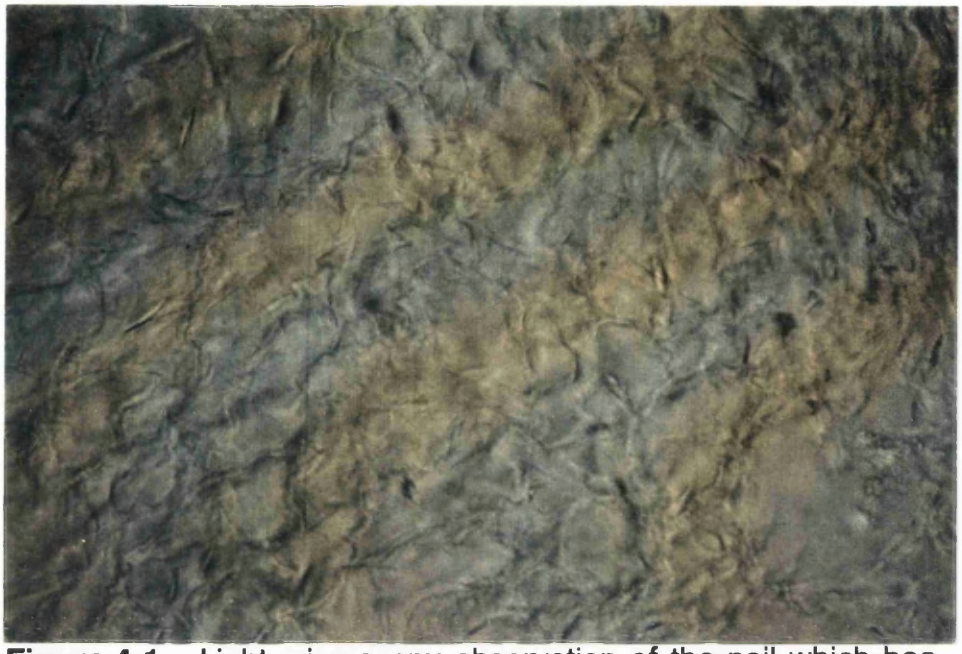


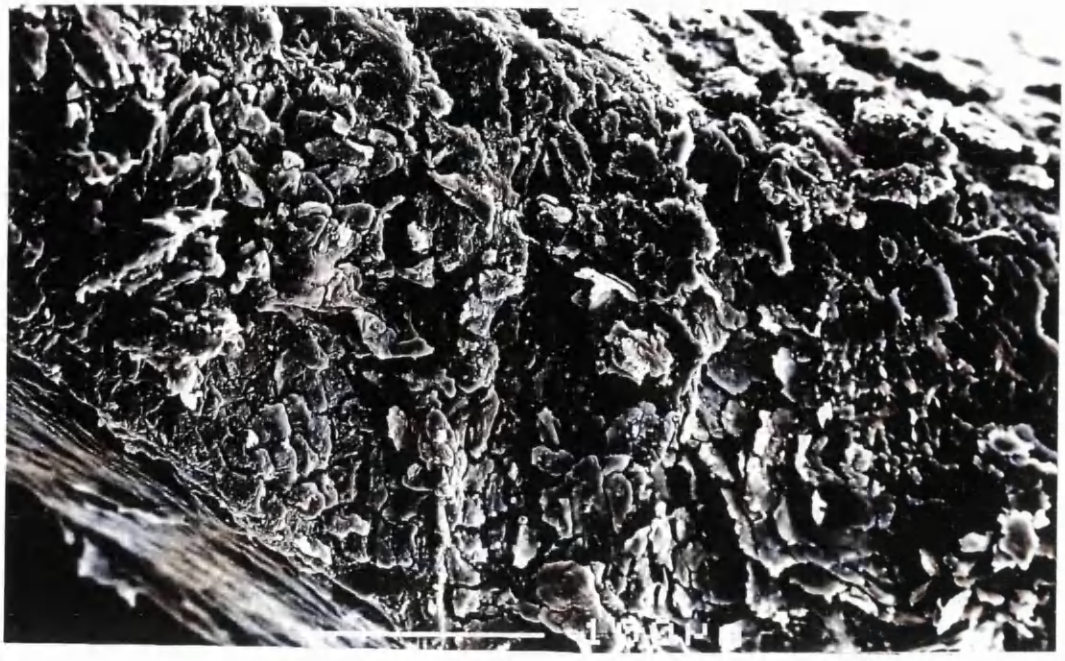
Figure 4:1 Light microscopy observation of the nail which has been digested with KOH. Note the regular close appearance of the lamellar cells X 400.

Figure 4:2a

Figure 4:2b

Figure 4:2 Scanning electron micrograph of a nail clipping (a) Section through the nail clipping. The dorsal nail surface appears at the top of the picture, followed by the intermediate zone. The most distal part, is the ventral side of the nail plate X 160 (b) the dorsal surface showing lamellar cells presenting a smooth tile – like arrangement X 1100 (c) palmar or ventral surface showing the irregular surface topography with deep grooves in the longitudinal axis X 540.

Figure 4:2c



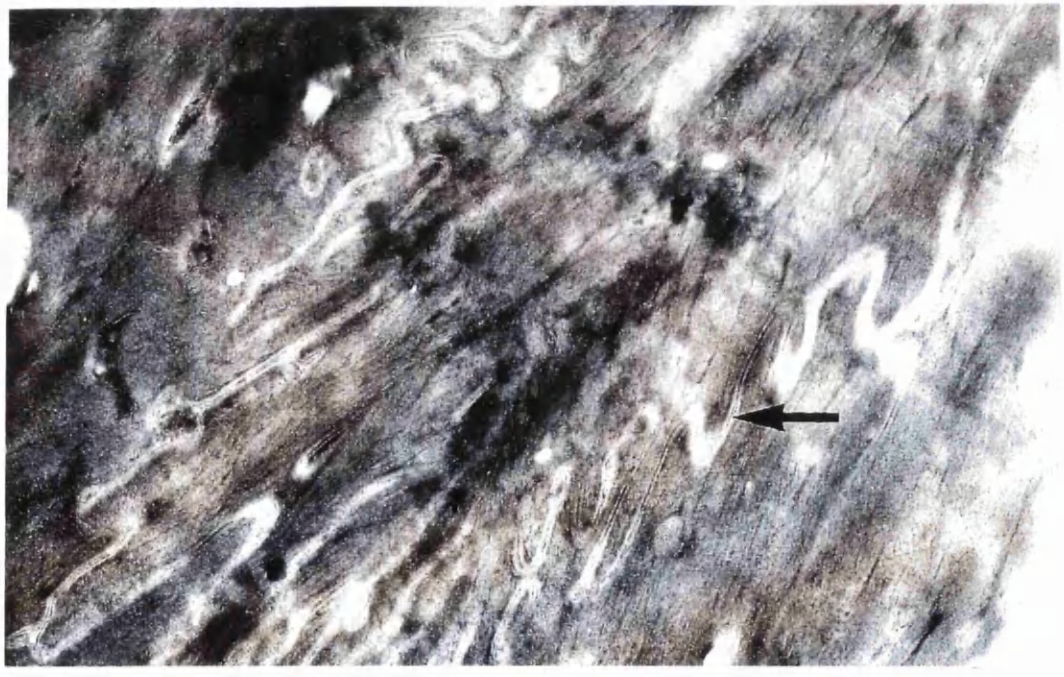


Figure 4:3 Transmission electron micrograph of fully hardened nail. Note the tortuous cell membranes with dense areas (arrows) which probably represent the attachment plaques of the desmosomes X 45000.

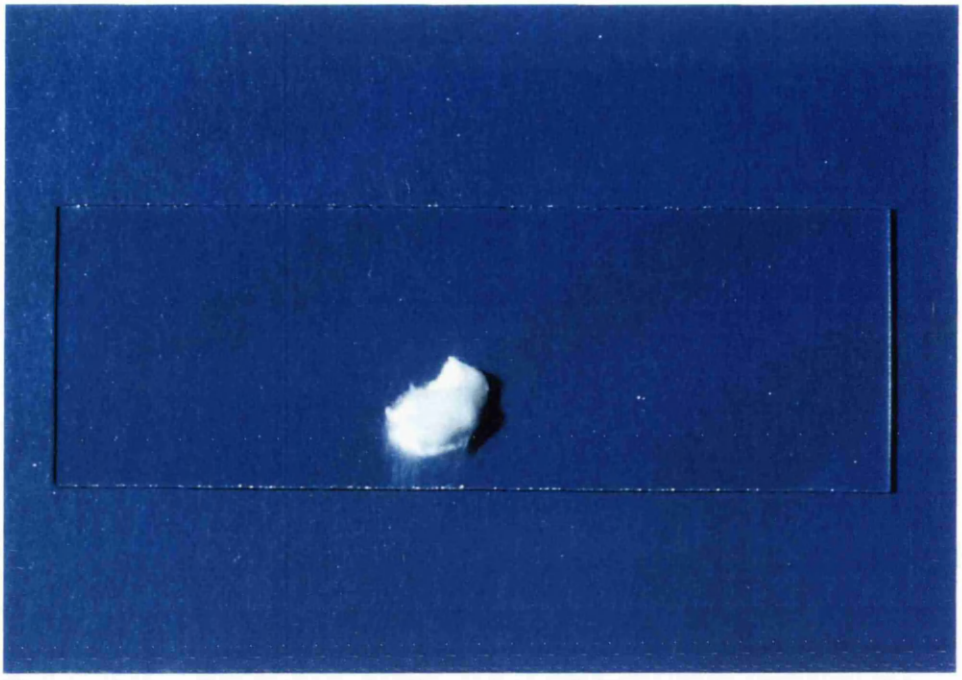


Figure 4:4 Gross examination of a nail fragment submerged in a mycelium of *T. mentagrophytes* after 7 days incubation.

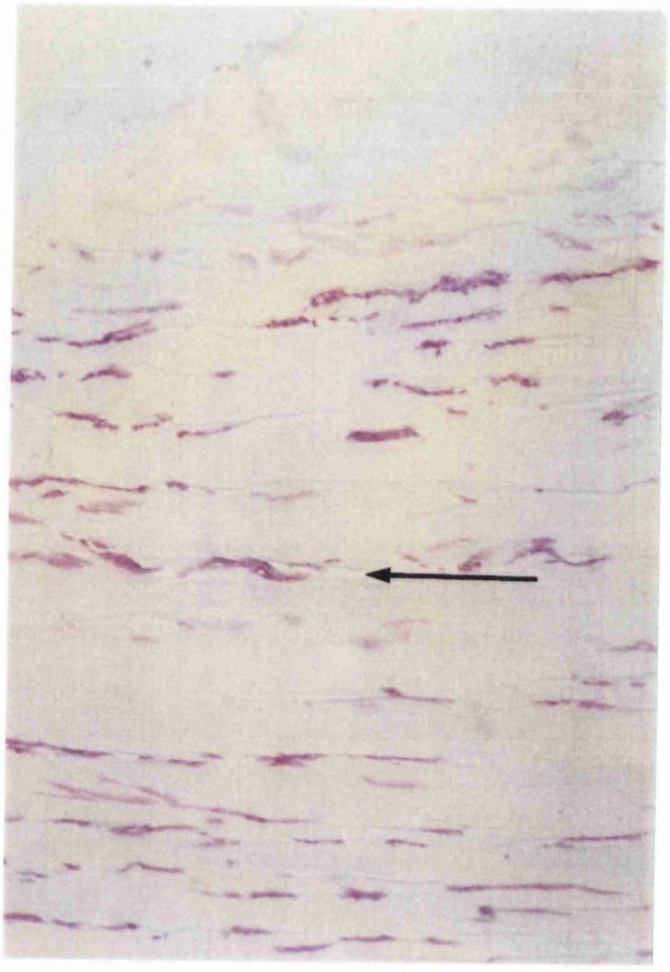


Figure 4:5 Transverse section of nail clipping. Note the fungal elements growing in parallel rows and within slits (arrow) in the nail X 400. PAS stain

Figure 4:6a

Figure 4:6b

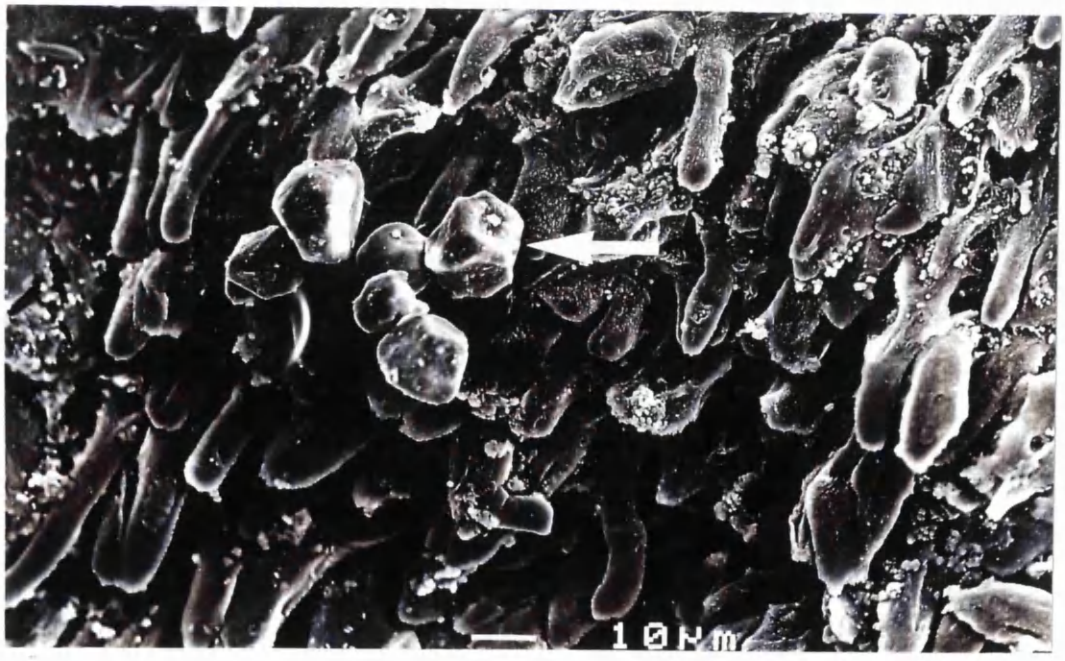


Figure 4:6 Scanning electron micrograph of *T. mentagrophytes* arthroconidia adhering to nail corneocytes (a) Two arthroconidia adhering on the ventral surface of the nail. Note the spherical shape and smooth outer cell wall of the arthroconidia. A depression is formed where they are adhering (arrow) X 3400 (b) arthroconidia adhering to tips of nail corneocytes on the ventral surface of the nail (arrow) X 1400 (c) bacteria seen adhering to the arthroconidium surface X 5000.

Figure 4:6c

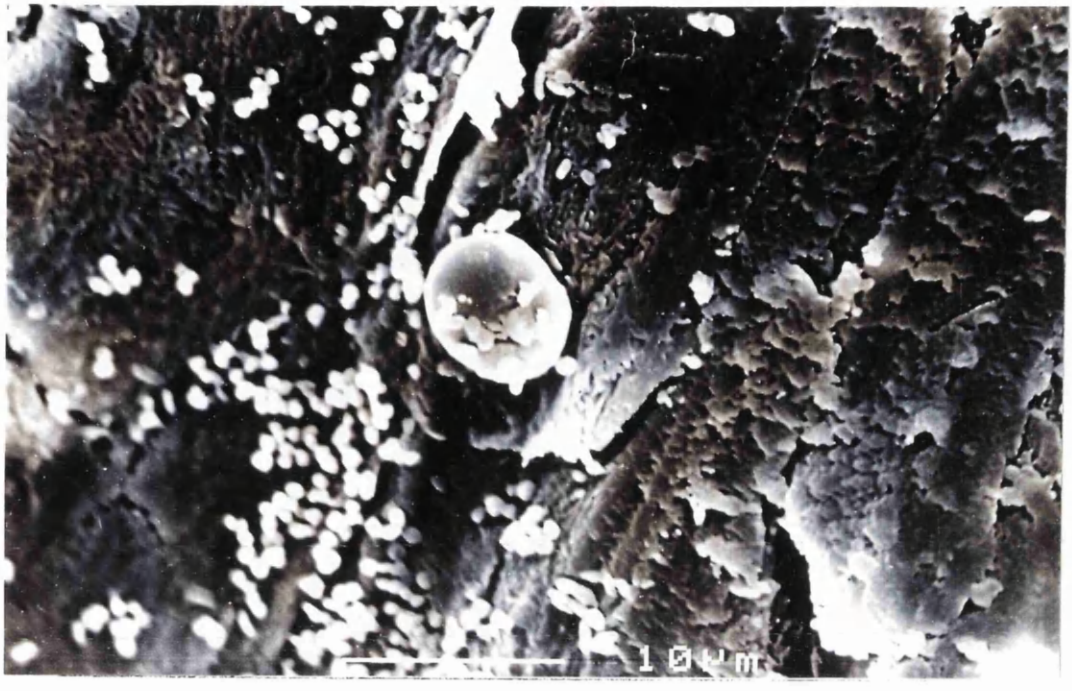


Figure 4:7a

Figure 4:7b

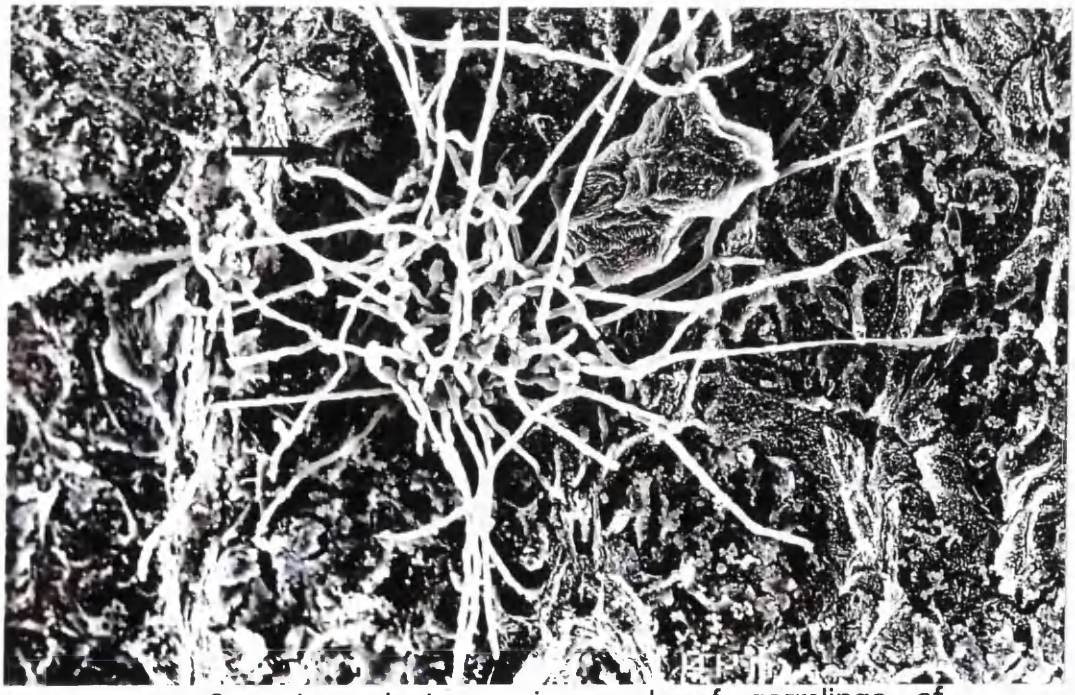


Figure 4:7 Scanning electron micrograph of germlings of *T. mentagrophytes* (a) Note multiple germ tubes arising from the parent arthroconidium and the tips are penetrating behind the corneocyte after 16h incubation X 2800 (b) a micro-colony is formed on the ventral surface of the nail after 24h incubation. Note the penetration of the nail by tips of germlings (arrow) X1100.

Figure 4:8a

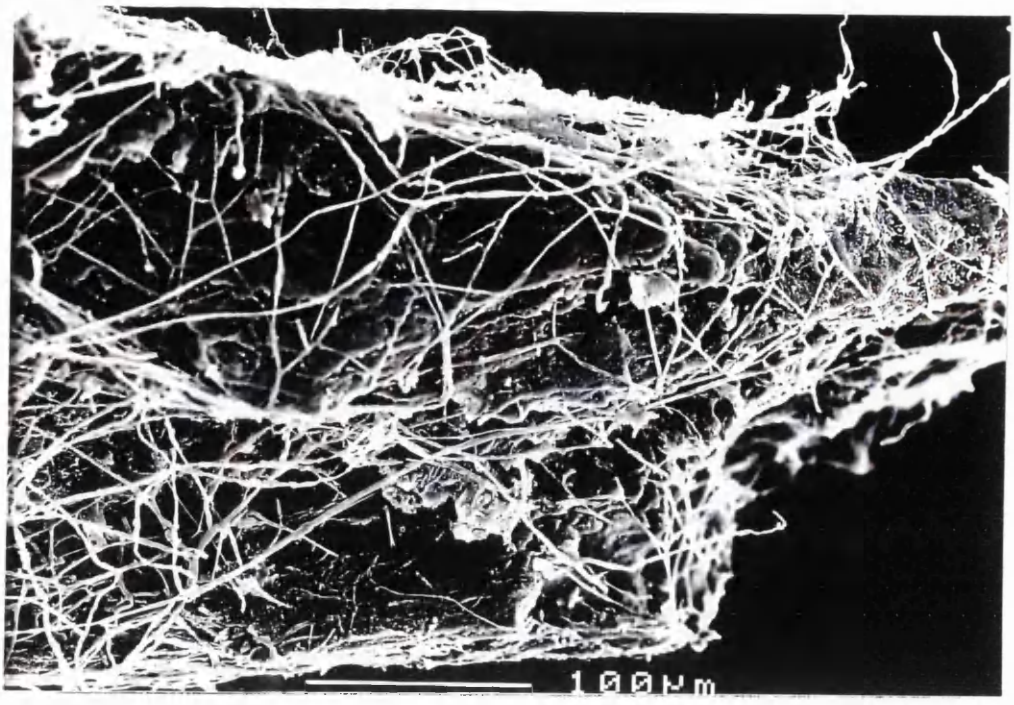


Figure 4:8b

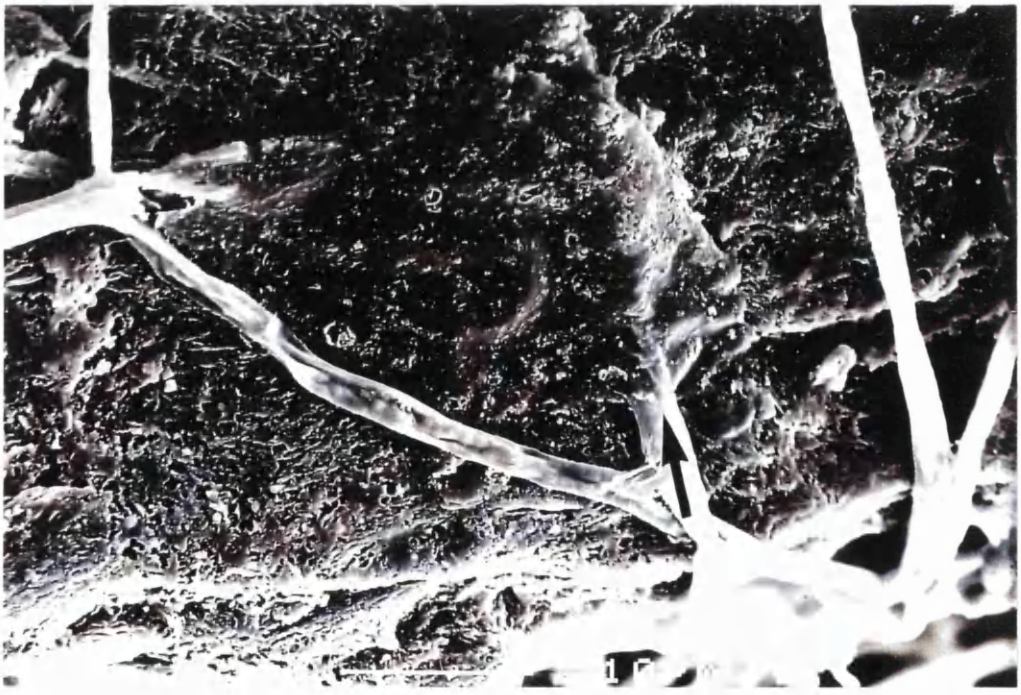


Figure 4:8 Scanning electron micrograph of a nail fragment (a) covered by a mycelial mesh of *T. mentagrophytes* after 72h incubation X 540 (b) a hyphae penetrating the nail surface (arrow) X 4400 (c) Note the close adherence of a hyphae to the surface, with a smooth surface and septa along the length of the filament (arrow) X 18000.

Figure 4:8c

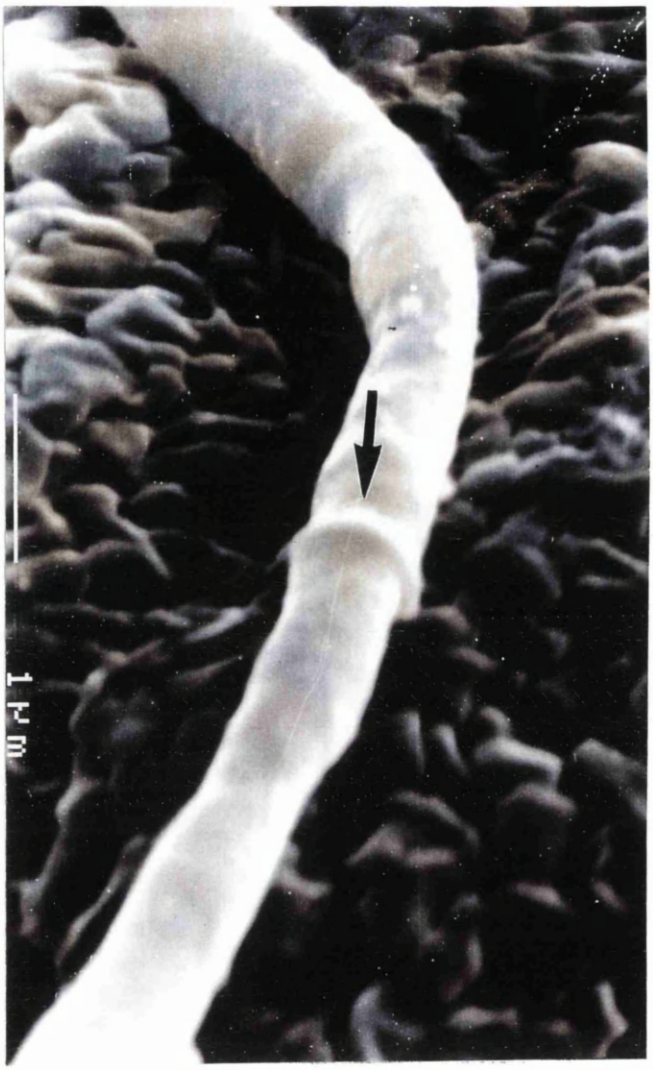


Figure 4:9a

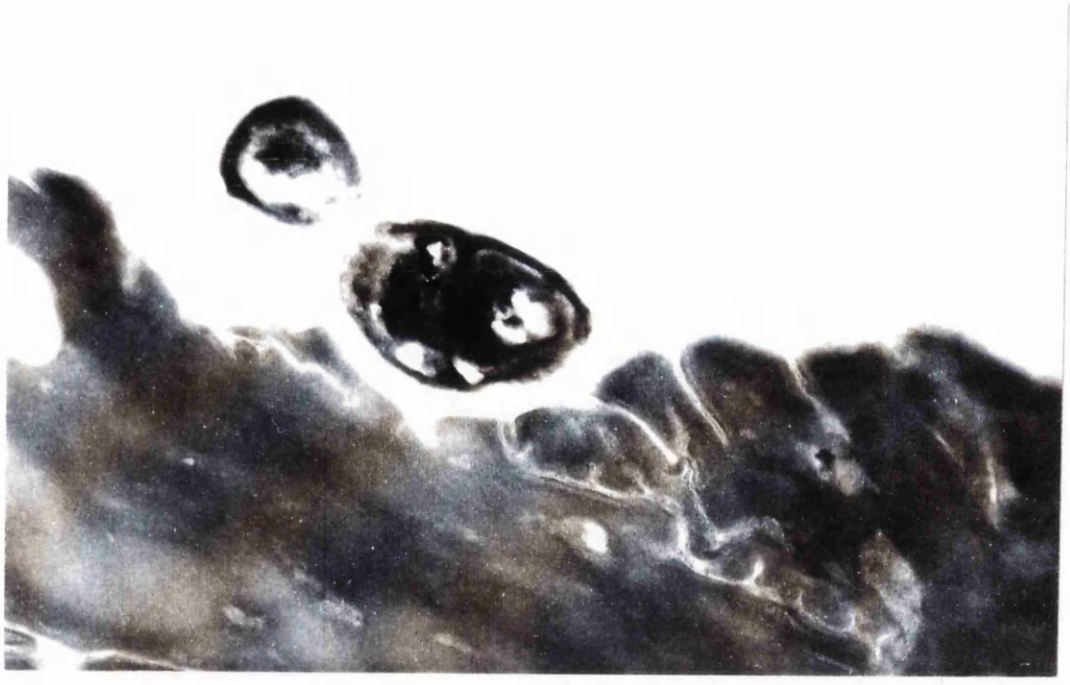


Figure 4:9b

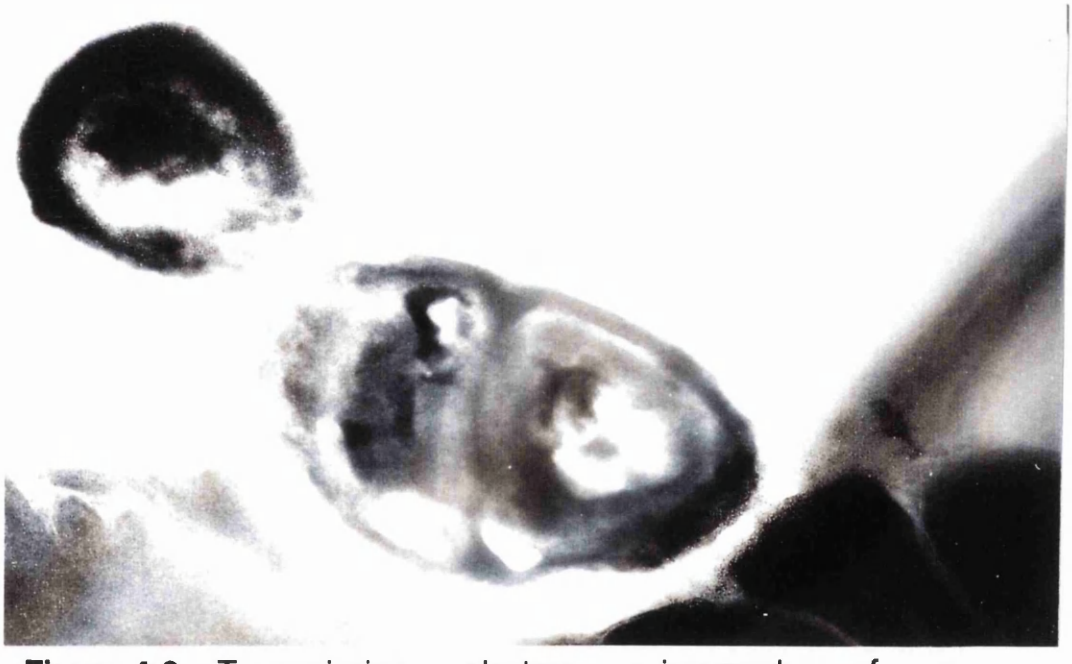


Figure 4:9 Transmission electron micrograph of a *T. mentagrophytes* arthroconidium adhering to nail corneocyte on the ventral surface of the nail (a) Note the depression formed where adherence is taking place X 45000 (b) the presence of a fibrillar–floccular material between the arthroconidial surface and tip of the nail corneocyte X 90000.

Figure 4:10 Transmission electron micrograph of *T. mentagrophytes* arthroconidia adhering to the nail surface. Note the penetration of the nail through the cervices (arrow) X3750.

Figure 4:11a

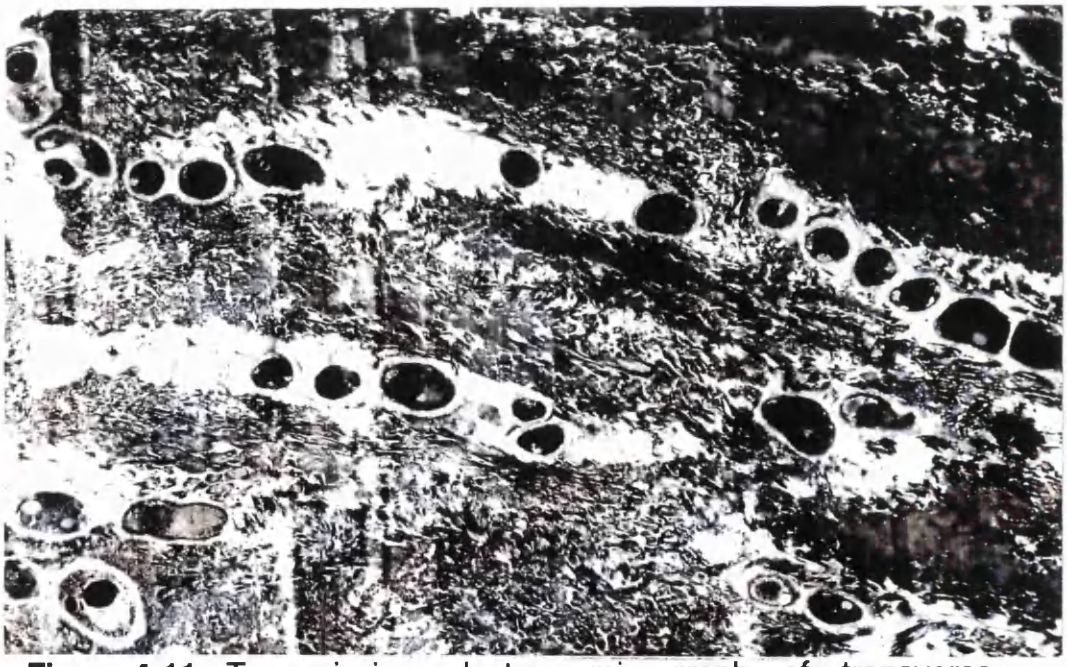


Figure 4:11 Transmission electron micrograph of transverse section through the nail after 2 weeks incubation (a) Note the chains of *T. mentagrophytes* arthroconidia penetrating through well formed channels which are lying parallel to each other X 4500 (b) multiple rows of arthroconidia, with keratin bundles lying in a disorganised way X 4500.

Figure 4:11b

Figure 4:12a

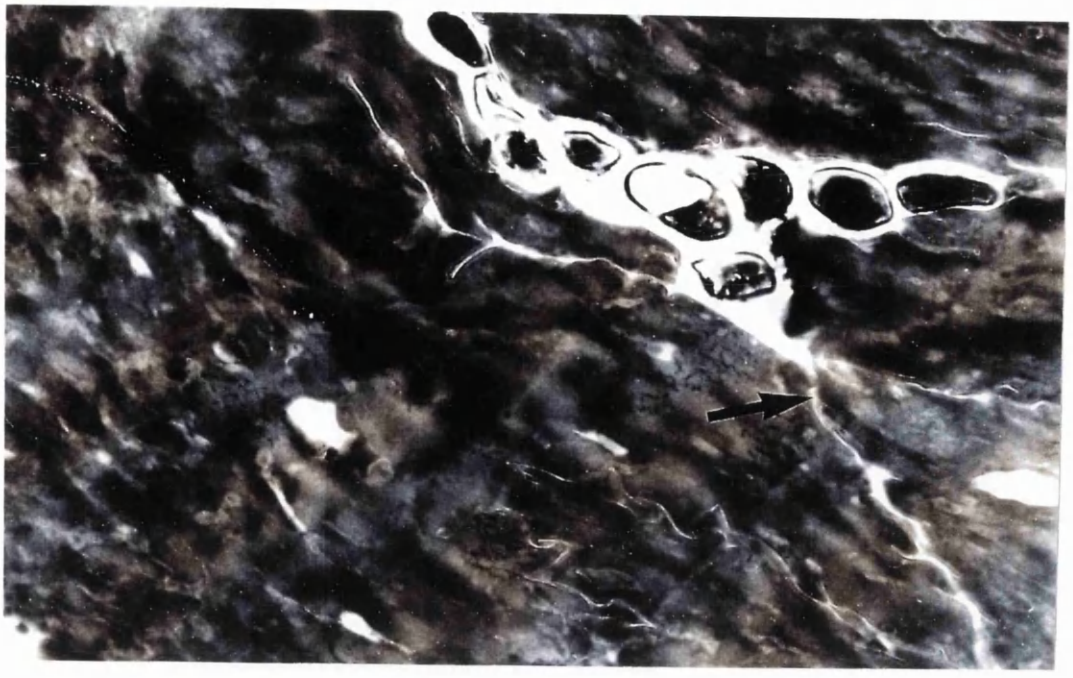


Figure 4:12b

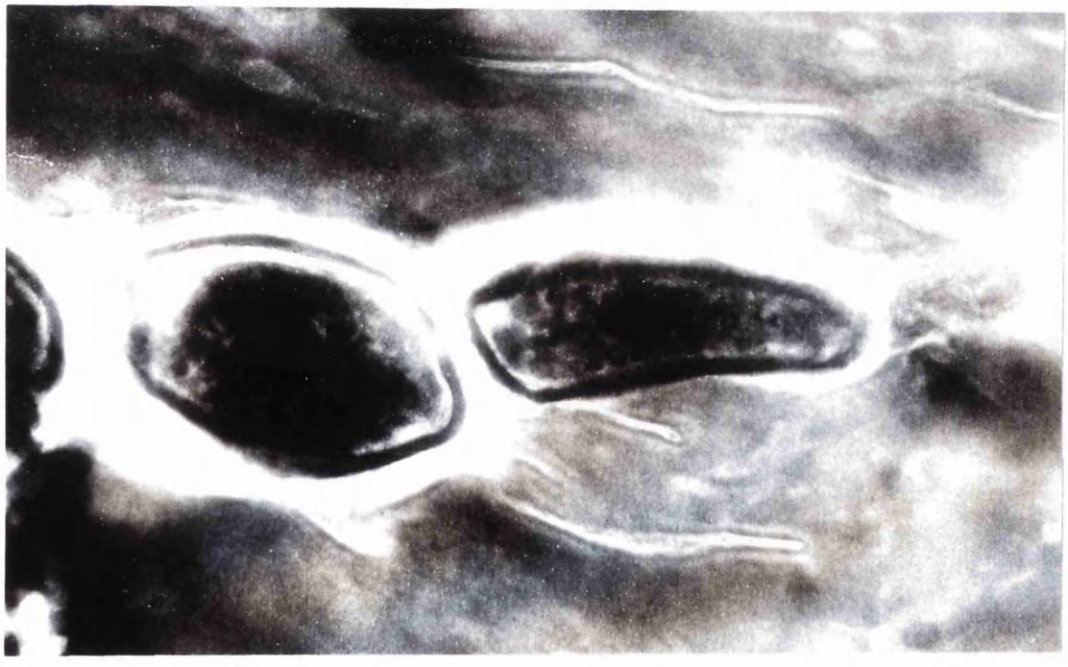
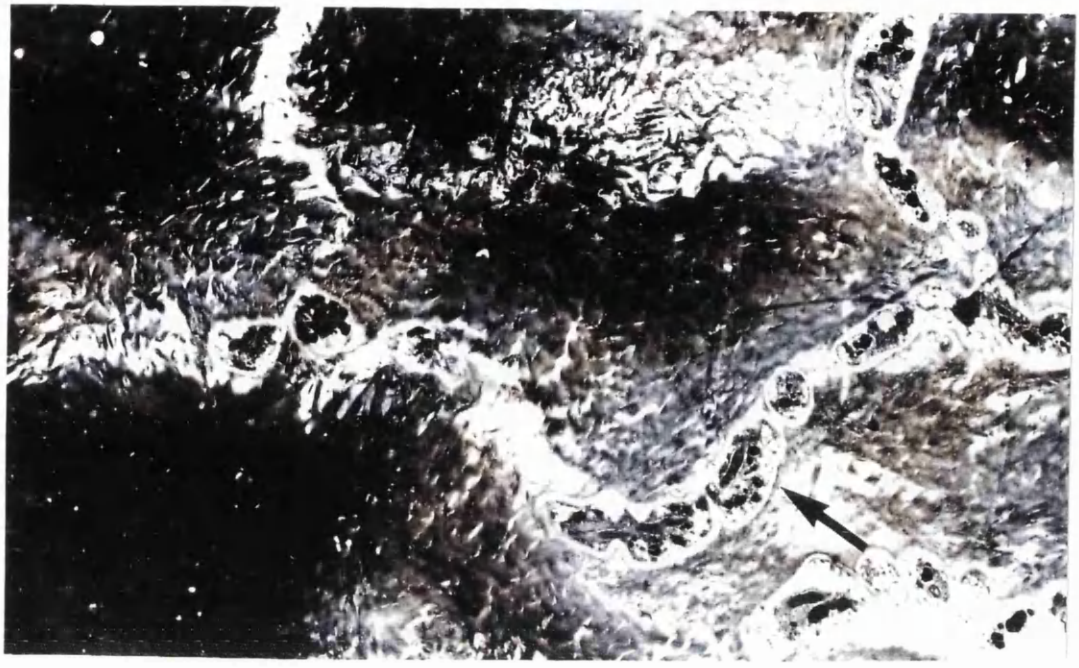


Figure 4:12 Transmission electron micrograph showing intercellular penetration of the nail by arthroconidia of *T. mentagrophytes* (a) Note the borders of nail corneocytes (arrow) X18000 (b) Note the clear zone around the fungal elements within the nail X 75000 (c) Separation of the cells at the intercellular junctions is observed with arthroconidia penetrating through the intercellular spaces (arrow) X18000. .

Figure 4:12c



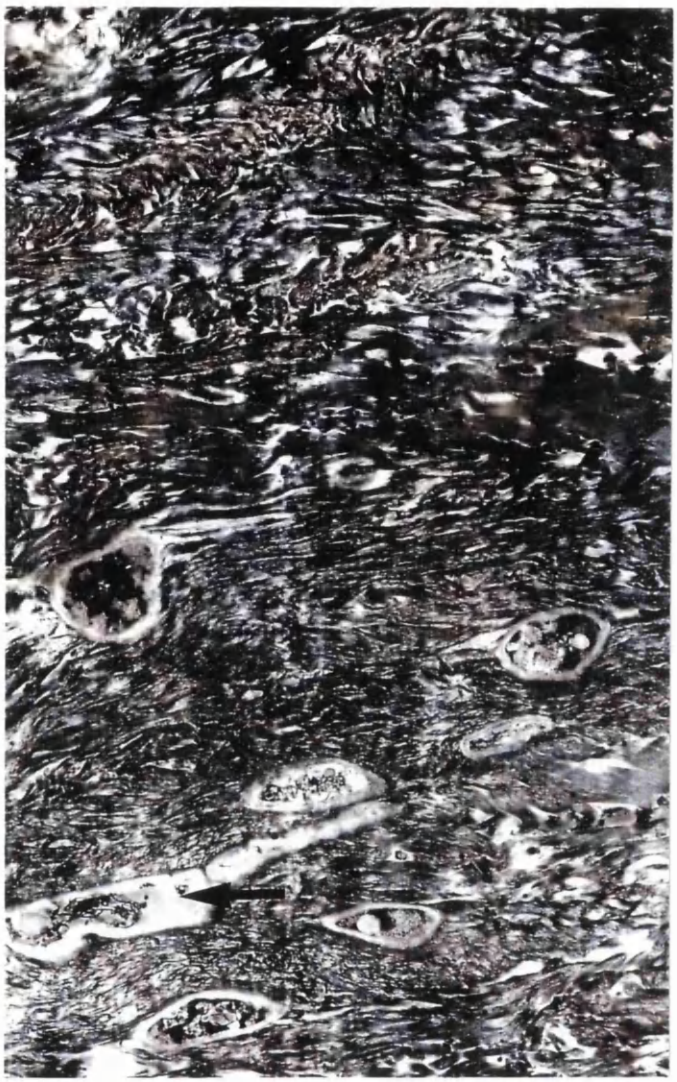


Figure 4:13 Transmission electron micrograph of a section through the nail. Note the penetration at different levels of the nail with a clear zone around the fungus (arrow) X 6000.

Figure 4:14a

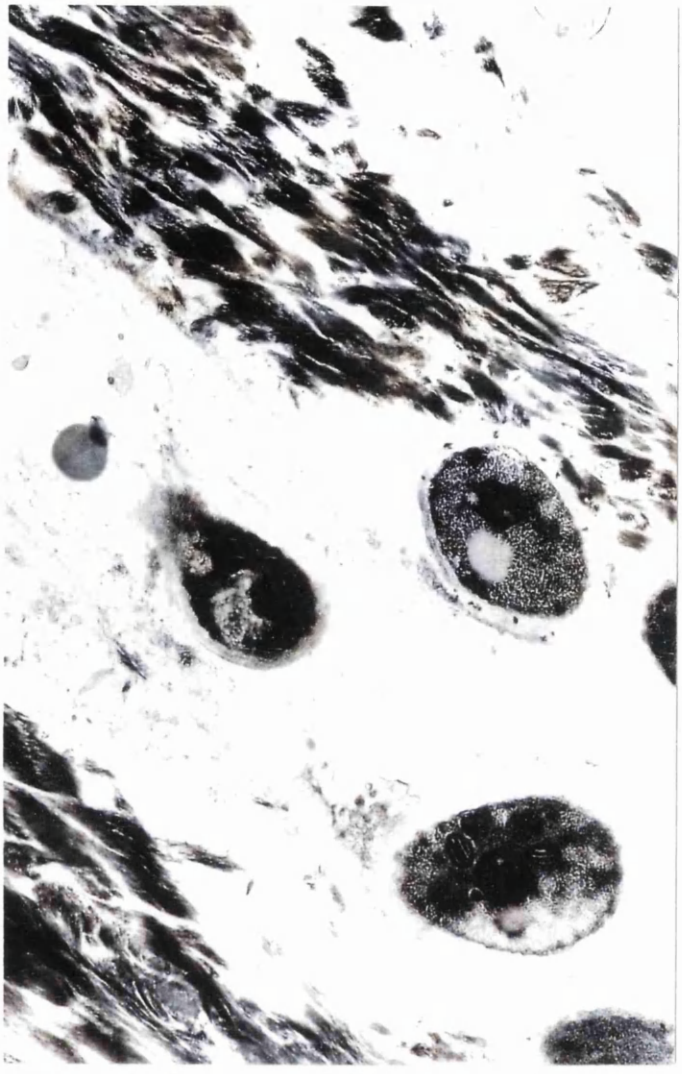
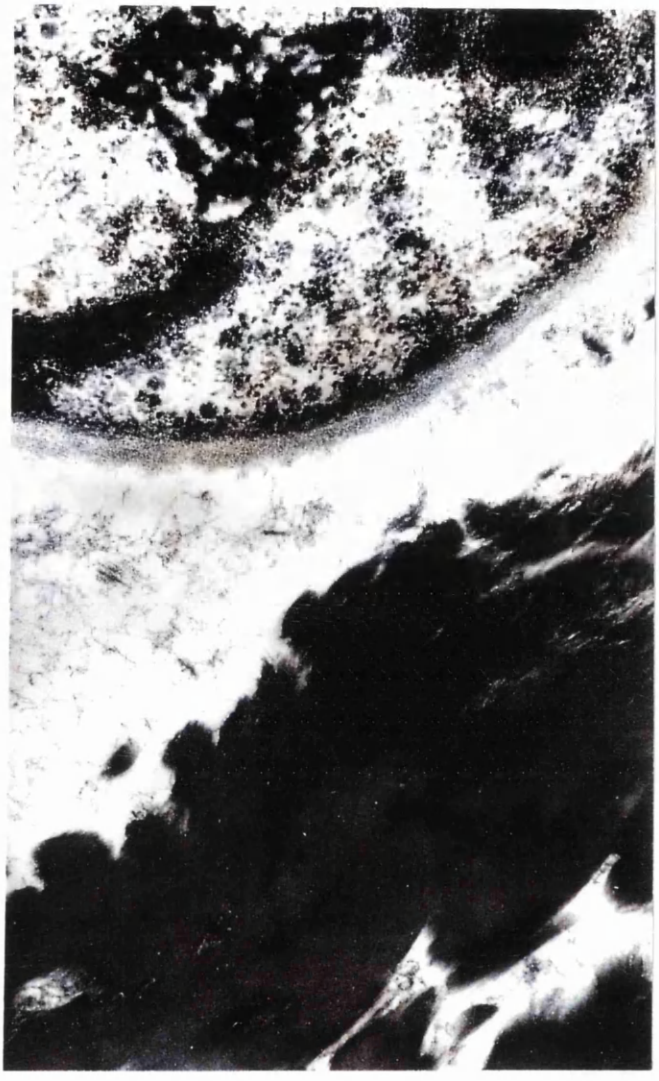


Figure 4:14 Transmission electron micrograph of *T. mentagrophytes* arthroconidia penetrating through the nail (a) Note the arthroconidia with double layered wall X 15000 (b) Note close adherence of the arthroconidia to the keratin bundles within the nail with a fibrillar material between their surfaces X 30000.

Figure 4:14b



penetration of the nail plate at all levels was seen. Cross sections of the channels were seen with a clear zone around the dermatophyte (Fig 4:13). At 3 weeks most of the nail substance was absent and mycelial fragments were seen. Detailed morphology of the arthroconidia was seen, the double layer wall, mitochondria, organelles and the golgi apparatus. Close adherence of the arthroconidia to the keratin bundles within the nail was apparent, with a fibrillar material between their surfaces (Fig 4:14).

4:6 Discussion

4:6:1 Normal nail ultrastructure

As distal subungual onychomycosis is the most frequent type of fungal nail infection, distal nail clippings were used in this study as the most appropriate substrate. They were easy to obtain and hence series of experiments could be performed. This part of the nail overlies the hyponychium. The lateral ends touch the lateral sulcus which have been postulated to be the main sites of entry of dermatophytes in the nail (Zaias, 1972a).

In the present study the normal structure of the nail showed a smooth dorsal surface with a ridged ventral surface. The cells showed digitations with the neighbouring cells. These findings are similar to those of Meyer and Grundmann (1984) and Forslind and Thyresson (1975). Meyer and Grundmann (1984) described the smooth dorsal surface of toe nails obtained from cadavers, on SEM presenting a "tile-like appearance". Forslind and Thyresson (1975) studied distal nail clippings similar to those used in the present study and considered the lamellar arrangement of cells in the nail plate of crucial importance for the strength of the nail plate. Both these studies described the irregular, ridged "wave-like" ventral or palmer layer which is in contact with the nail bed and allows interdigitations with the similarly shaped

nail bed. Meyer and Grundmann (1984) described this layer consisting of fibres similar to that present in the nail bed. The polygonal appearance of corneocytes in the hyponychium and the parallel ridges of the intermediate layer described by Meyer and Grundmann (1984) are similar to those observed in this study.

In the present study the process of nail invasion took place through the ridges and crevices on the ventral surface, through the parallel ridges on the lateral edges and by penetration of the hyphae through and in between the corneocytes on the dorsal surface. *T. mentagrophytes* can either invade the ventral layer and then involve the intermediate layer or directly the dorsal layer as seen in white superficial onychomycosis.

4:6:2 Growth of dermatophytes on nail

Adherence and germination of arthroconidia and growth of germ tubes was observed on nails in the absence of any added nutrients. The nails were fully covered by growth, and by three weeks were friable or had disappeared. On gross examination growth was seen on all of the nails which had been inoculated. It is known that dermatophytes are keratinophilic fungi; that is, they grow on keratinous substrates. Dermatophytes, particularly of the genus *Trichophyton,* are known to grow very well in human nails and thus nail serves as an excellent medium for their growth. It is said that *T. mentagrophytes* assimilates nail keratin (Alkiewicz, 1964). There have been many studies trying to examine the growth of dermatophytes on keratinous substrates and to establish that they grow on and digest keratin *in vitro.* Mathison (1964) thought that the keratinophilic ability of dermatophytes was due to their capacity to form perforating organs. Page (1950) studied keratin digestion by *Microsporum gypseum* using cow horn, finger nails, wool and hair as substrates. He observed that the horn particles ultimately disappeared and that human nails underwent a similar digestive fate. Hairs were also penetrated with again,

evidence of digestion. Based on these findings he suggested that *M. gypseum* and as well as other dermatophytes were capable of digesting keratin. Vanbreuseghem (1952) studied the growth of over one hundred different strains of dermatophytes on hair, found that every strain grew on hair, though the *in vitro* growth was different from the *in vivo* growth, proving the strong keratolytic power of these fungi. Callus, hair and nail are all degraded by dermatophytes *in vitro* (English 1963). Raubitschek and Maoz (1957) and Raubitschek (1961) hypothesised that dermatophytes grew in the nail by digesting the intercellular cement or living on the non-keratins. Such a sequence of events lead to the break up of nails by mechanically breaking apart the keratinized cells rather than digesting them.

Zaias (1972a) found that *T. mentagrophytes* was a more active destroyer of nail tissue than *T. rubrum*. The actual mechanism of nail destruction was not clear, but it was speculated that a mechanical separation of nail lamellae rather than true keratinolysis was the cause. Sulphitolysis due to sulphite excretion by dermatophytes, leading to cleaving of the disulphide bonds of keratin has been observed by Kunert (1972a). The denatured keratin is then easily degraded by the dermatophyte proteinases. Wawrzkiewicz *et al.* (1991) suggested that the process of degradation of keratin in its natural sources seems to be due to a combination of mechanical and enzymatic action by the fungus. The disappearance of nail fragments in this study could be due to a combination of mechanical degradation and the proteolytic effect of proteinases produced by the fungus.

4:6:3 Nail invasion by dermatophyte fungi

In the present study nail invasion by arthroconidia of *T. mentagrophytes* was observed. Early penetration of the nail was seen on the ventral surface, followed by hyphae penetrating through and in between the corneocytes on the

dorsal surface. With time, all layers of the nail were invaded with chains of arthroconidia growing through well formed channels in parallel layers.

Adherence and germination of arthroconidia to corneocytes has been discussed in Chapter 1, section 1:3:2. Raubitschek and Maoz (1957) showed that the growth of *T. mentagrophytes* and *T. rubrum in vitro* consisted purely of hyphae in nail tissue. Page (1950) also showed this phenomenon for *M. gypseum*. Alkiewicz (1964) described that the fungus formed chains of spores in the nail plate during its growth, and that the growth in the nail plate was directional and took place in the intercellular spaces. He suggested that the observed empty spaces and channels were due to corrosion through tissue. Clinically this is seen as "transverse net" white threads with a transverse pattern (Alkiewicz, 1948) and "springy net" as described by Sowinski (1963) which could be seen with a drop of cedar oil on naked eye examination.

Since early penetration of the nail plate takes place from the ventral layer, arthroconidia were inoculated onto this surface. Small germ tubes, micro-colonies and penetration of nail from the ventral surface was seen on transverse sections. Here, the fungus was observed to grow in a vertical direction. The growth pattern of the fungus indicates that it grows in the spaces between cells. Meyer *et al.* (1981) looked at mycotic nails under SEM in four patients who had *T. rubrum* and *T. mentagrophytes* infections. They found that the dermatophyte entered the nail from the ventral surface and that the hyphae were lying along the direction of the cells. They also noticed intercellular and direct penetration of the corneocytes by the hyphae and holes in the nail which appeared similar to those seen in the present study. Meyer *et al.* (1981) suggested that the holes detected were possibly due to an enzymatic action of the dermatophyte and could represent part of the tunnels seen in the sections of mycotic nails.

The ventral nail surface is derived from the nail bed (Johnson *et al.*, 1991). It's production is continuous along the length of the nail. (Johnson and Shuster, 1990). The junctions between cells in the lower plate are more flexible

when compared to the tight junctions in the dorsal nail plate (Baden and Kvedar, 1991). Most dermatophyte species invade the middle and ventral layers of the nail plate adjacent to the bed where the keratin is comparatively soft, and it is in close proximity to the living cells below (English, 1976). In the present study initial penetration of the nail was from the ventral surface. Furthermore, there were many similarities between the growth of the dermatophyte *in vivo* and *in vitro* in the nails.

With longer incubations all three layers of the nail were invaded which could be explained by the fact that the fungus could have damaged the sulphydryl links (Kunert, 1972a), thus rendering the process of invasion easier. The dorsal nail plate is the hardest part of the nail and has a high calcium content being formed from the upper nail matrix. It is rarely invaded except directly in superficial white onychomycoisis (Zaias, 1972b). Haneke (1991) showed that if fungal hyphae can be demonstrated histologically in all of the layers of the nail plate at the free edge of the nail, this may be taken as evidence of penetration of the proximal matrix.

4:7 Conclusion

In this study growth of dermatophyte fungi was observed on the nails. Germination of arthroconidia as well as germ tube extension was observed. There were similarities between the growth of the fungi on the nails and stratum corneum. The findings of nail invasion in the present study are similar to those based on descriptions of patients with mycotic nails except that all three layers were invaded by the fungus which rarely occurs *in vivo*. Previous studies have employed nutrients to enhance nail penetration by dermatophyte fungi. In this study nail infection was established in the absence of these factors. It seems that dermatophytes invade the nail by a combination of mechanical and chemical factors. This is the first comprehensive study of the process of nail invasion *in vitro* by dermatophyte fungi in the absence of any nutrients. A new

in vitro model for investigation of nail invasion by dermatophyte fungi is described. It could be adapted to study the invasion of nails by non-dermatophyte fungi and bacteria. This model could be expanded to study antifungal drug activity in nails.

Chapter 5

Inhibitory effect of terbinafine on the invasion of nails by dermatophyte fungi

5:1 Summary

The application of nail fragments as a model for the study of antifungal drug activity against dermatophyte fungi was investigated. A strain of *T. mentagrophytes* and low concentrations of terbinafine were used. The effect of terbinafine on adherence and germination of arthroconidia and growth of germlings on nails was assessed by gross examination, light and electron microscopy. The study showed that pre-exposure of nail fragments to terbinafine concentrations (0.01–10 mg/L) inhibited fungal growth and acted as a barrier to dermatophyte invasion. Damaged arthroconidia and distorted hyphae were observed by scanning electron microscopy. This *in vitro* model provides an alternative system for studying the activity of antifungal agents in nail.

5:2 Introduction

Antifungal drugs may penetrate the nail plate by one of two routes. Firstly, the drug may be incorporated into the matrix and then penetrate the nail plate as a band of drug moving along with the nail as the nail grows (Zaias, 1985). Adequate systemic therapy with antifungal drug then acts as a barrier against further proliferation of the fungus in the affected nail and causes a nail free of infection to grow over the surface of the digit (Zaias and Drachman, 1983). Alternatively, the drug could diffuse into the nail plate from the nail bed. In this case fungicidal levels of the drug would be reached in the distal nail plate at an early stage after therapy (Matthieu *et al.*, 1991; Munro *et al.*, 1992). The allylamine derivative terbinafine has also been shown to penetrate via the nail bed. The agent appears to be fungicidal with rapid penetration into the nail plate. Penetration through the ventral nail significantly contributes to the rapid action of terbinafine; and the fact that short duration therapy is effective in onychomycosis (Goodfield *et al.*, 1992; Munro *et al.*, 1992).

The penetration of antifungal drugs through the nail plate is a crucial requirement for successful treatment of onychomycosis. The thickness of the nail and its relatively compact construction make it a formidable barrier to the entry of topically applied drugs. The rate of diffusion across the nail plate is inversely proportional to the nail thickness. Furthermore, the nail plate barrier property appears to be intact for long periods of aqueous immersion (Walters *et al.*, 1981). The nail plate exhibits behaviour similar to that of hydrogel of high ionic strength. Topical bioavailability can be enhanced by decreasing the formulation pH thereby increasing drug solubility (Walters *et al.*, 1985).

The nail is prone to attack by a host of micro-organisms, including fungi, some of which invade the living tissues of the nail bed and nail fold and others invade the nail plate itself (English, 1976). Dermatophyte nail infection is a chronic condition and a source of inconvenience, pain and discomfort and the infection contributes to and perpetuates the reservoir of dermatophyte fungi.

There are few satisfactory experimental models for the study of the activity of antifungal agents in nails, and the morphological changes in dermatophytes following exposure to antifungal - treated nails. Arthroconidia are the morphological forms of dermatophytes most likely to play a major role in the persistence and spread of dermatophytosis and have not previously been used in experimental models of onychomycosis.

5:3 Aim of the Study

The aim of the study was to examine the barrier effect of low concentrations of terbinafine against nail invasion by germlings of *T. mentagrophytes* in a model using arthroconidia to represent the parasitic infections propagule of dermatophyte fungi. This would enable us to understand how antifungal drugs penetrate the nail plate and their mode of action in onychomycosis.

5:4 Materials and Methods

5:4:1 Organism and stock cultures

One strain of *T. mentagrophytes* was used in this study. This strain was the same as described in Chapter 4, section 4:4:1. Subcultures were maintained on GPA and incubated at 28°C for 10 days to obtain an optimal yield of microconidia.

5:4:2 Preparation and production of arthroconidia

Arthroconidia were produced and prepared to a concentration of $5x10^4$ arthroconidia per ml as described in Chapter 2, sections 2:4:3 and 2:4:4. Viability of single arthroconidia was determined as described in Chapter 2, section 2:4:4.

5:4:3 Preparation of nail fragments

Distal fragments of normal human nails were used for this study as described in Chapter 4, section 4:4:3. Nails were also obtained from healthy volunteers who were not undergoing antifungal therapy. The nails were autoclaved for 5 min at 5lb pressure. A series of nails were not autoclaved.

5:4:4 Preparation of terbinafine solutions

Solutions of terbinafine were prepared as described in Chapter 3, section 3:4:4. A 100 mg/L stock solution was prepared. By serial dilution a concentration range of (0.001–10 mg/L) was prepared.

5:4:5 Assessment of antifungal activity

Nail fragments were saturated with terbinafine concentrations either for 15 min or 3h, inoculated with 50µl of arthroconidial suspension placed in a humidified atmosphere and then incubated at 28°C for varying time intervals. A series of nails saturated with terbinafine were washed twice in distilled water and then inoculated with arthroconidial suspension. Similarly, nail fragments from other individuals saturated with terbinafine and then inoculated were also prepared. Gross examination was carried out and then potassium hydroxide mounted nails were examined under the light microscope (x400). For control purposes nails were soaked in distilled water and appropriate volumes of DMSO. Electron microscopy was carried out to observe ultrastructural changes in nails, arthroconidia and germ tubes.

5:5 Results

5:5:1 Gross examination

On gross examination, growth was visible on control nails by 4–5 days of incubation at 28°C. Growth was luxuriant and gradually covered the nail fragments. On terbinafine exposed nails, growth was initially slow and only at concentrations of 0.001 mg/L and below was there growth comparable to controls. At concentrations of 0.01 mg/L and above growth when seen was much less than controls and the nails were not necessarily fully covered. At concentrations of 0.1 mg/L and above there was some degree of growth inhibition seen on all nails. Generally at a drug concentration of 1 mg/L and above there was no visible growth on the nails and also occasionally at 0.1 mg/L (Fig 5:1). Even though the growth was not seen on gross examination this did not exclude growth on light microscopic examination. KOH mounts of all nails were performed before discarding the nails as positive or negative for

the presence of hyphae. Some nails with no visible surface growth showed the presence of hyphae and a mycelium on examination of KOH mounts. Nail fragments washed following exposure to terbinafine showed more surface growth as compared to non-washed fragments but the concentration of drug inhibiting growth did not differ. Growth of the fungus was more rapid on finger nails, normal and terbinafine exposed as compared to toe nails.

5:5:2 Light microscopy

Scrapings from all nail fragments were mounted in 20% (w/v) KOH, a coverslip placed on them and then examined under a x400 eye piece. On controls, germination of arthroconidia took place, and well formed branching hyphae were seen growing on the surface of the nail fragments. By 72h hyphae had completely covered the nail fragment forming a mycelium mat on the nail. On gross examination growth was not visible at this stage. Growth on terbinafine exposed nail was inhibited and was concentration dependent. At high concentrations, growth was not seen. On nail fragments exposed to terbinafine at concentrations of <0.001 mg/L a similar appearance was seen as in controls. Concentrations of >0.01 mg/L gave a varying degree of growth inhibition and non-involvement of the nail surface was seen (Fig 5:2). The hyphae were not as well formed as in controls; these tended to be swollen and showed segmentation with arthroconidia formation occurring as early as 72h. The growth of hyphae on terbinafine exposed nails was slower.

On terbinafine exposed finger nails, following 15 min exposure, and using a mycelial suspension, growth was inhibited at concentrations of >0.25 mg/L on finger nails, and on toe nails at >0.5 mg/L. At concentrations of >2 mg/L growth was not seen (Table 5:1). Using an arthroconidial inoculum, growth was inhibited at concentrations of >0.125 mg/L on finger nails, and on toe nails at >0.25 mg/L. At concentrations of >2 mg/L growth was not observed (Table 5:1). Using a mycelial suspension and 3h exposure of the nails to the

drug, growth was inhibited at concentrations of >0.01 mg/L on finger nails and 0.03 mg/L on toe nails. At concentrations of >1 mg/L growth was not seen. While with an arthroconidial inoculum growth was inhibited on finger nails at concentrations of >0.01 mg/L, and on toe nails at a concentration of >0.03 mg/L. At concentrations of >0.25 mg/L growth was not observed (Table 5:1).

Exposure Time (*x*,*y*)	Inoculum	MIC Range
FN*x*	(i)	0.25 – 2 *n*
FN*x*	(ii)	0.125 – 2 *n*
FN*y*	(i)	0.01 – 1 *n*
FN*y*	(ii)	0.01 – 0.25 *n*
TN*x*	(i)	0.5 – 2 *n*
TN*x*	(ii)	0.25 – 2 *n*
TN*y*	(i)	0.03 – 1 *n*
TN*y*	(ii)	0.03 – 0.25 *n*

Table 5:1 MIC values describing the effects of exposure to terbinafine. FN = finger nails, TN=toe nails, *x*=15 min, *y*=3h and *n* = mg/L. Mycelial suspension (i) and arthroconidial suspension (ii). Each experiment was carried out in triplicate.

5:5:3 Electron microscopy

On scanning electron microscopy where nail fragments were exposed to terbinafine (0.01– 1 mg/L) and then inoculated with arthroconidia, adherence and germination was seen. The arthroconidia were seen to have a rough surface with layers exfoliating off. Some had single or multiple pores on their surface (Fig 5:3). Arthroconidia were seen to form a depression where they were adhering to the ventral surface of the nail. The hyphae appeared dilated with swollen tips and were disrupted into fragments throughout the terbinafine

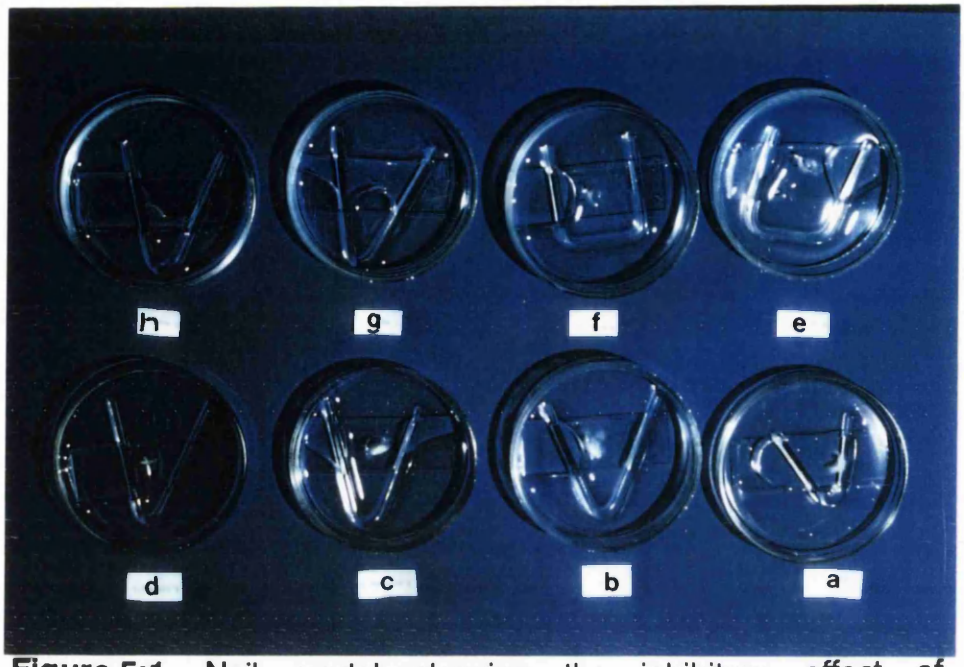


Figure 5:1 Nail model showing the inhibitory effect of terbinafine at concentrations greater that 0.001 mg/L.
a = control, b = DMSO, c = 0.001mg/L, d = 0.01mg/L, e = 0.1mg/L
f = 1mg/L, g = 2mg/L, h = 10mg/L.

Figure 5:2a

Figure 5:2b

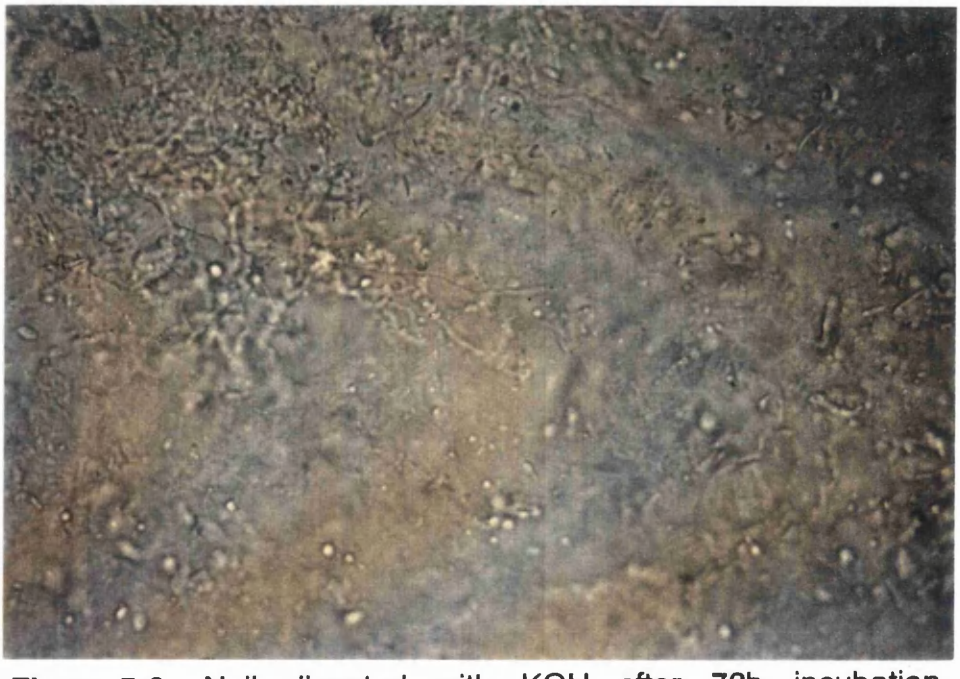


Figure 5:2 Nail digested with KOH after 72h incubation showing (a) Control hyphae of *T. mentagrophytes* covering most of the nail surface X 250(b) nail fragment exposed to 0.01 mg/L terbinafine showing growth inhibition and non–involvement of the nail surface X 250.

Figure 5:3a

Figure 5:3 Scanning electron micrograph of a *T. mentagrophytes* arthroconidium (a) Adhering to the ventral surface of the terbinafine–exposed (0.01 mg/L) nail fragment. Note the formation of pores (arrow) in the arthroconidium wall X 17000. (b) an arthroconidium adhering to the surface of terbinafine – exposed (0.01mg/L) nail fragment. Note the rough surface with layers exfoliating off X 30000.

Figure 5:3b

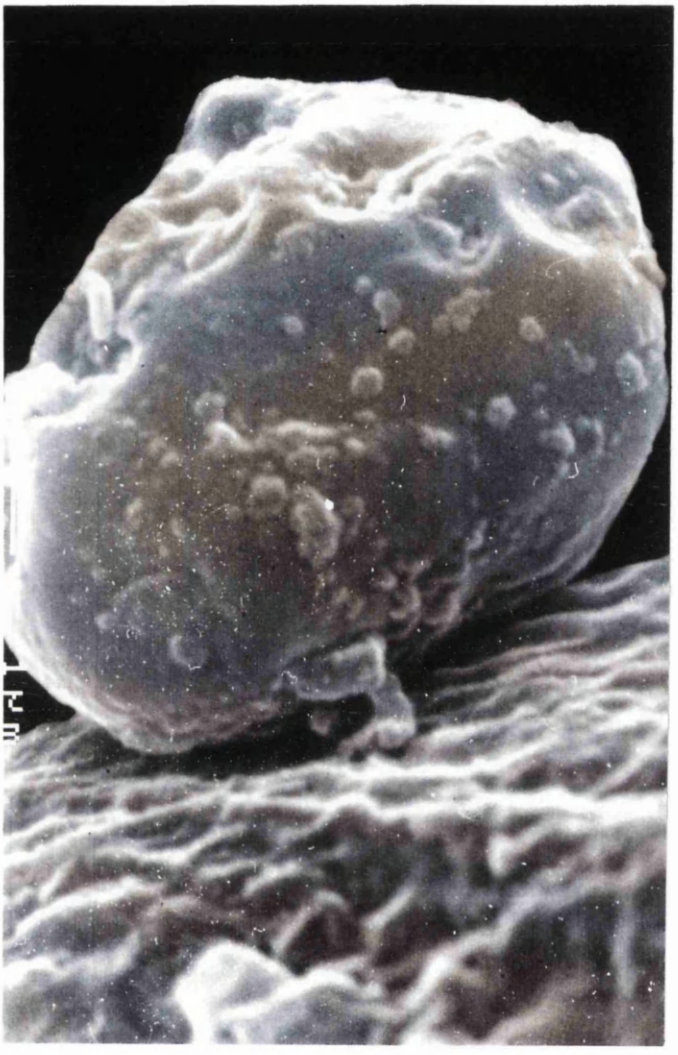


Figure 5:4a

Figure 5:4b

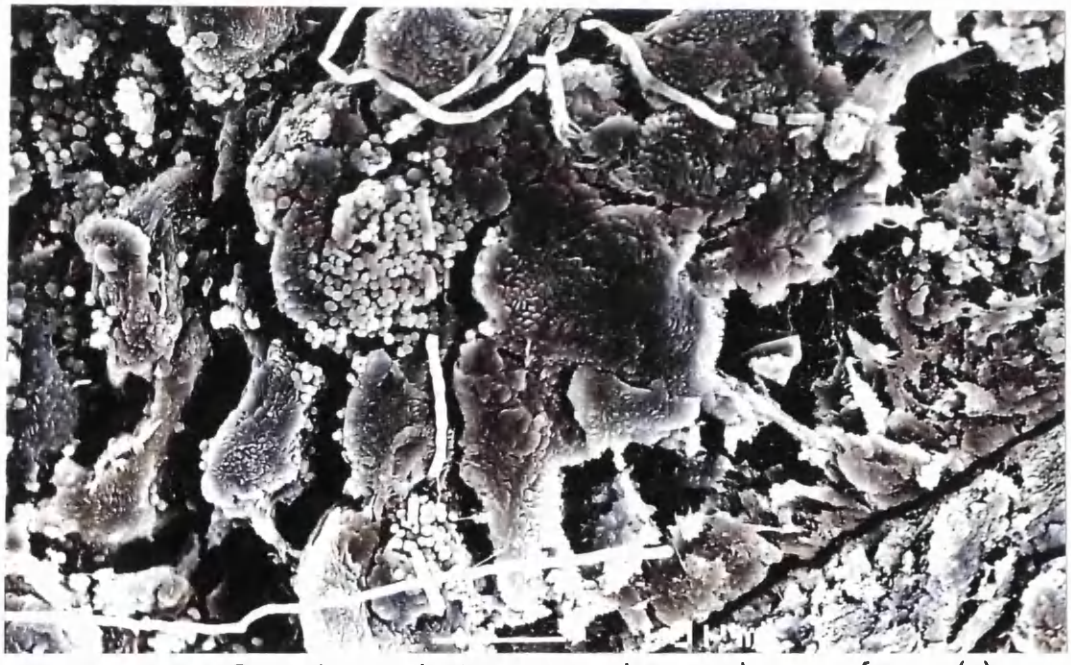


Figure 5:4 Scanning electron micrograph of (a) *T. mentagrophyte* mycelium on terbinafine–exposed (0.01 mg/L) nail. Note the disruption of the mycelium with areas of sparing on the nail surface X 900 (b) hyphae on terbinafine–exposed (0.01 mg/L)/nail. Note the fragmentation and collapse of hyphal elements on the ventral surface of the nail X 3000 (c) fragmentation of fungal hyphae X 16000.

Figure 5:4c

concentration range (0.01–1 mg/L) with inhibition of hyphal extension being evident. At higher concentrations collapsed hyphae were seen (Fig 5:4). No evidence of nail penetration was seen at concentrations above 1 mg/L. On terbinafine–exposed nails there were areas where no growth was seen. Transmission electron microscopy of terbinafine–exposed nail fragments did not show any structural change. At 1 mg/L there was evidence of intercellular penetration in terbinafine–exposed nails. No evidence of nail penetration was seen on TEM at concentrations of 2 mg/L and above.

5:6 Discussion

5:6:1 Barrier effect of terbinafine against nail invasion by *T. mentagrophytes*

Pre–exposure of nail fragments to low concentrations of terbinafine inhibited fungal growth and acted as a barrier to dermatophyte invasion. Longer exposure to terbinafine was more effective in inhibiting growth. Terbinafine appeared to diffuse rapidly through the nail plate and remain in an active form as seen by its fungicidal activity in this model. Normally during treatment of onychomycosis, orally administered antifungals penetrate the nail plate via the nail matrix. Adequate systemic therapy then acts as a barrier against further proliferation of the fungus in the affected nail and causes a fungus–free nail to grow over the surface of the digit (Zaias and Drachman, 1983). A method for assessing antifungal drug effectiveness in onychomycosis by measuring the normal nail plate growth following initiation of systemic therapy with griseofulvin and ketoconazole was also used (Zaias and Drachman, 1983).

Recently itraconazole and terbinafine have been shown to penetrate via the nail bed (Mathieu *et al.*, 1991 Munro *et al.*, 1992). The latter penetration route may significantly contribute to the response to oral antifungal therapy in

onychomycosis. Since in most fungal infections of the nail organ, the fungi are located under the nail surface and in the nail bed (Scher and Ackerman, 1980) and grow into the nail plate. Therefore the penetration of the drug through the nail plate is a crucial requirement for successful treatment. An insufficient penetration of the drug through the nail plate, which is an extremely rigid structure with poor metabolism, accounts for the lack of therapeutic success. The continuous production of ventral nail provides a large and previously unsuspected route of rapid access for drugs into nails operating along the whole of their length (Johnson and Shuster, 1990). Munro *et al.* (1992) showed that the ventral nail, which contributes up to 30% of nail mass, as it grows along the bed provides rapid access of drugs to the site of distal disease. This could explain the rapid action of terbinafine and the fact that short duration therapy is effective in onychomycosis.

Terbinafine is an effective treatment for dermatophyte onychomycosis. Research on nail pharmcokinetics with terbinafine has shown that the drug rapidly diffuses into the nail plate from the nail bed, achieves fungicidal levels and persists in the nail plate following cessation of therapy (Finlay, 1992). Fungicidal levels of terbinafine are detected in distal nail clippings as early as four weeks after commencing treatment. Dykes *et al.* (1990) showed that the levels of terbinafine ranged from 0.1–2.89 ng/mg nail. They also showed that following repeated administration there was no more accumulation of the drug in the nail. The MIC for terbinafine against dermatophyte fungi *in vitro* is 0.001 – 0.02 μg/ml (Ryder and Mieth, 1992).

The results show that a longer exposure of the nails to terbinafine was more effective in inhibiting growth. Terbinafine is detected in the nails as early as three weeks following commencement of therapy. The speed of detection indicates that the drug must be diffusing through the nail, as the rate of new nail growth was not fast enough to account for the presence of the drug as a result of being taken up into newly formed nail at the proximal nail bed. With the longer exposure possibly more drug was being absorbed in the nail. A lower

MIC was seen for finger nails and with the longer 3h exposure of nail fragments to terbinafine, corresponding with the clinical observation that finger nails mycoses respond more rapidly to the drug than toe nails mycoses and generally need only half the treatment time. This may be due to the faster rate of growth of finger nails and an enhanced incorporation of the drug in finger nails. The fingers have a better blood supply than the feet (Davies *et al.*, 1967) and hence finger nails may consequently receive more terbinafine. With longer exposure possibly more drug was being absorbed in the nail fragments.

Studies with alkanol permeabilities pattern in general reflect nail plate behaviour and shows how other organic substances of low molecular weight might penetrate the nail (Walters *et al.*, 1983). For penetration purposes the hydrated nail plate behaves as a membrane of hydrogel of high ionic strength to polar and semipolar alcohols. At extreme hydrophobicity there is increased permeability, indicating the emergence of a functional lipid pathway (Walters *et al.*, 1983). The nail plate hydration plays a role in increasing the rate of permeation, probably through increasing the diffusibility of these compounds. There are differences in the rate of water transpiration between the nail plate and the stratum corneum, nail being 10 times more permeable. The nail plate is up to 1000 times more intrinsically permeable to water than the stratum corneum. Franz (1992) in experiments using amorolfine has shown that the rate of absorption was vehicle dependent and the use of penetration enhancers resulted in higher rates of absorption. It has been shown that DMSO increases the penetration rate in the skin, but has shown little promise as acceleration of nail plate permeability (Kligman, 1965). Walters *et al.* (1985) showed that the ionic form of miconazole dissolves as early as the free base in the nail, thus emphasising the importance of decreasing the formulation pH thereby increasing drug solubility and hence topical bioavailability. Stuttgen and Bauer (1982) looked at the penetration of antimycotics, econazole, oxiconazole and dimethylmorpholinohydrochloride in the human skin and nails. They found that the crucial factor in treating nail infections was the fungicidal concentration

achieved by the drug within the nail, which was capable of killing the arthroconidia.

5:6:2 Morphological changes in *T. mentagrophytes* arthroconidia and germlings following exposure to terbinafine-exposed nails

The results showed that pre-exposure of the nails to terbinafine did not prevent adherence or germination of arthroconidia, but was inhibiting the growth of germlings and was also fungicidal. Arthroconidia were observed to be more sensitive to low concentrations of the drug in the nails than mycelial fragments. The morphological findings in arthroconidia and germ tube on terbinafine-exposed nails are similar to those described on the stratum corneum by Rashid *et al.*, (1993). In that study a much higher concentration of terbinafine was used. Nishiyama *et al.* (1991) described dilated hyphae with swollen tips using a lower concentration of terbinafine incorporated in culture media. Recently, Vismer *et al.* (1993) observed morphological changes in the fungal elements present in nail tissue from patients with onychomycosis while being treated with Lamisil. They described distorted hyphae, and blown up hyphal segments and hyphal remnants. These findings are very similar to those observed in the present study. The possible mode of action of terbinafine on the growth forms of *T. mentagrophytes* has been discussed in Chapter 3, sections 3:6:1 and 3:6:2.

5:7 Conclusion

In this study germination of arthroconidia was seen on terbinafine exposed nails but hyphal growth was inhibited. A short exposure of the nails to low concentrations of terbinafine acted as a barrier against nail invasion by *T. mentagrophytes.* It was shown that arthroconidia are more sensitive to terbinafine than mycelial fragments and morphological changes were observed

in arthroconidia and germlings. It is likely that terbinafine is somehow deposited in the nail and acts as a barrier to the further invasion of this tissue by dermatophyte fungi. The increased sensitivity of arthroconidia to this drug could explain the marked efficacy of terbinafine in tinea ungium as the dermatophyte mainly exists as arthroconidia in the nails. This *in vitro* model provides an alternative system for studying the activity of antifungal agents in nail and the morphological changes in dermatophytes following exposure to antifungal-treated nails.

Chapter 6

Invasion of hair follicles by dermatophyte fungi and the inhibitory effect of terbinafine

6:1 Summary

A novel *in vitro* model for the study of hair invasion by dermatophyte fungi and the effects of antifungal drugs was developed. One strain of *T. mentagrophytes* and hair obtained by micro-dissection and plucking were used. Arthroconidia were inoculated onto the hair with and without terbinafine. In the absence of terbinafine growth of the fungus was seen on the hair follicle. Growth was observed to begin at the shaft end and extend along the hair shaft towards the bulb area. The inner root sheath provided a good substrate for fungal growth. Initially, the cuticle formed a barrier to fungal penetration of the hair. Eventually, germlings of *T. mentagrophytes* were seen to penetrate under the cuticle and in between the layers of cuticular cells to invade the cortex. There was no evidence of intracellular growth; fungal elements were seen intercellularly. There were similarities between the findings in this study of the process of hair invasion by dermatophyte fungi and that in the natural disease. Where hair was in the presence of low (0.01–10 mg/L) concentrations of terbinafine, inhibition of growth was seen and morphological changes were observed in the arthroconidia and germ tubes. This *in vitro* model provides a promising experimental system for studying the process of hair invasion by dermatophyte fungi and the effects of antifungal drugs in hair and their mode of action in tinea capitis.

6:2 Introduction

Tinea capitis or dermatophyte infection of hair is mainly of two types, ectothrix and endothrix. In ectothrix infection arthroconidia can be seen outside the keratinized hair shaft while in endothrix infection arthroconidia formation occurs within the hair shaft (Kilgman, 1955). The affected hair tissues have been morphologically studied using light microscopy, scanning, and transmission electron microscopy (Tosti *et al.*, 1970; Okuda *et al.*, 1989, 1991).

However, ultrastructural findings of the parasitic form of *T. mentagrophytes* in the hair tissue and the pathologic changes of the affected hair structure are not sufficiently known. The stages by which detached hairs are attacked by keratinophilic fungi are (i) cuticle lifting (ii) cortical erosion (iii) production of penetrating organs and (iv) colonization of the medulla (English, 1963). The ability to invade hair *in vitro* is a property of the keratinophilic fungi in general but various species differ in the way this is accomplished. It has been found that the direction of invasion and the pathological role of the fungal elements within the hair apparatus are significantly different between the species of fungi (Okuda *et al.*, 1991).

Baxter and Mann (1969) observed the invasion of human hairs *in vitro* by three keratinophilic fungi, namely, *T. mentagrophytes, T. rubrum and T. ajelloi.* Differences were seen in the penetration and digestion of cuticular cells by these three species of fungi. The early stage in the colonization of human hair by *T. mentagrophytes* involves the production of flattened, branched fronds, the "eroding mycelium" which seems to be common to all species of keratinophilic fungi but the "perforating organs" are formed only by, for example, *T. mentagrophytes* and *T. ajelloi.* Based on studies of hair invasion described by Mercer and Verma (1963) and Baxter and Mann (1969) there is evidence that the process of hair invasion involves enzymatic breakdown. Hsu and Volz (1975) observed complete digestion of human hair due to enzymatic lysis by *T. terrestre.* A specific keratinase has been isolated from *T. mentagrophytes* which is capable of digesting hair (Yu *et al.*, 1969). Kunert and Krajci (1981) studied the degradation and digestion of human hairs by *M. gypseum in vitro* and suggested that along with the action of enzymes, mechanical action of the hyphae was also found in the cuticle. Experimental studies of hair penetration by dermatophytes are few (Mercer and Verma, 1963; Raubitschek and Evron, 1963; Kaaman and Forslind, 1985), and arthroconidia have not been previously used in experimental studies of hair infection. Neither are there any satisfactory experimental models for detailed study of the invasion of hair follicles by

dermatophyte fungi and the effects of antifungal drugs in hair and their mode of action in tinea capitis.

The lack of understanding of the process of hair invasion by dermatophyte fungi and how antifungal drugs work in tinea capitis has been caused in part by the lack of good *in vitro* models. As long as the "reconstituted hair" *in vitro* does not exist, hair research continues to depend on culture models of isolated dermal and epidermal components of the hair follicle. Several *in vitrc* models or organ culture systems have been developed for the study of hair follicle biology. Recently Philpott *et al.* (1990) reported the maintenance and growth of human hair follicles *in vitro*. They observed that hair follicle length increased time dependently and that [^{3}H] thymidine uptake, [^{14}C] leucine uptake and hair keratin synthesis occurred in cultured hair follicle. The increase in hair follicle length could be attributed to the production of a keratinized hair shaft. By careful micro-dissection it is possible to obtain undamaged hair follicles which grow *in vitro* at an *in vivo* rate. Using this model screening experiments with antifungal drugs, with possible influence on human hair invasion by dermatophyte fungi might be carried out to a larger extent.

It is assumed that systemically administered antifungal agents are most probably incorporated into the hair shaft and hair root sheath as well as excreted by sebaceous glands and sweat glands, forming a chemical barrier for the fungi and thus gradually removing them from the hair in the course of hair growth. The allylamine derivative, terbinafine is an effective short-term therapy for tinea capitis (Haroon *et al.*, 1992). It reaches high concentrations in the sebum (up to 45.1 μg/ml) and high levels are achieved in and around the hair follicle, reaching 2.6 μg/g of tissue. It is also delivered to the hair follicle by sebum. The drug continues to concentrate in sebum up to two days after discontinuation of therapy. The high level of drug in the sebum is beneficial for the treatment of fungal infections of the hair follicle as it combines diffusion through the skin to the affected areas with high concentration in the hair follicle (Faergemann *et al.*, 1990).

6:3 Aim of the study

This study was undertaken to investigate the ability of *T. mentagrophytes* arthroconidia to adhere and morphologically transform on hair follicles cultured *in vitro* and to observe the process of hair colonization and invasion by germlings of *T. mentagrophytes*. Also to explore the possibility of utilising this hair model to study the inhibitory effect of low concentrations of terbinafine against hair follicle invasion by germlings of *T. mentagrophytes*. This study would enable us to understand the process of hair invasion by dermatophyte fungi in detail and how antifungal drugs work in tinea capitis.

6:4 Materials and methods

6:4:1 Organism and stock cultures

One strain of *T. mentagrophytes* was used in this study . This strain was the same as described in Chapter 4, section 4:4:1. Subcultures were maintained on GPA and incubated at 28°C for 10 days to obtain an optimal yield of microconidia.

6:4:2 Production and Preparation of arthroconidia

Arthroconidia were produced and prepared to a concentration of $5x10^4$ arthroconidia per ml as described in Chapter 2, sections 2:4:3 and 2:4:4.

6:4:3 Micro-dissection and maintenance of hair follicles in organ culture

Using a stereo microscope, scalpel and tweezers, anagen hair follicles were microdissected from fresh skin pieces of normal human scalp. Hair follicles were dissected at the dermo-subcutaneous fat interface and were

maintained for 4 days at 37°C in Dulbecco's modifed eagle's medium supplemented with 2mMw/v L-glutamine and gentamycin (50 µg/ml). Macroscopic observation revealed continued viability as shown by increased growth of maintained follicles with the appearance of a hair shaft. Terminal hairs were plucked from the scalp, and anagen roots cut with a scalpel just above the adherent outer root sheath.

6:4:4 Preparation of terbinafine solutions

Solutions of terbinafine were prepared as described in Chapter 3, section 3:4:4. A 100 mg/L solution was prepared and by serial dilution a concentration range of 0.01-10mg/L was prepared.

6:4:5 Inoculation of hair follicles

Dissected human terminal hair follicles were washed twice in carbon dioxide independent tissue culture medium (Appendix) and then placed onto nucleopore filter membranes floating on 2 ml of CO_2-independent tissue culture medium. Similarly plucked anagen hair follicles were also placed on a nucleopore filter membrane floating on CO_2-independent medium with and without terbinafine. A 5µl inoculum of a suspension containing $5x10^4$ arthroconidia per ml was inoculated onto the hair shaft end of the follicles. The petri dishes containing hair follicles were incubated for varying lengths of time at 28°C. Hair follicles set up as above but without arthroconidia inoculated on them were used as controls.

6:4:6 Assessment of hair invasion and antifungal activity

Gross examination was done to assess fungal growth. Intact specimens were examined daily under the microscope and photographed. For light

microscopy hairs were set up as in section 6:4:5, then examined by placing under a light microscope. For toluidine staining hairs were fixed in glutaraldehyde and processed as for transmission electron microscopy described in Chapter 2 section 2:4:9. Sections 1μm in thickness were cut and stained with toluidine blue.

For electron microscopy hair follicles were set-up as in section 6:4:5 and after growth had proceeded for the desired time they were fixed and processed for scanning and transmission electron microscopy as in Chapter 2, section 2:4:9.

For assessing antifungal activity, hair follicles were exposed to terbinafine concentrations (0.01–10 mg/L) and light microscopic examination was carried out and toluidine blue sections of these hair were examined under the light microscope (x400). Scanning electron microscopy was carried out to observe ultrastructural changes in the hair, arthroconidia and germlings.

6:5 Results

6:5:1 Hair follicle invasion by *T. mentagrophytes* arthroconidia

6:5:1:1 Light microscopy

On examination under a light microscope, micro dissected hair follicles showed an increase in length, which was confirmed with the appearance of a keratinized hair shaft. At 4 days dissected hair follicles showed a fully formed hair shaft emerging from the connective tissue sheath (Fig 6:1). Whereas plucked hair follicles (anagen) had retained the bulb area. Arthroconidial germination was visible on inoculated follicles by 16h of incubation at 28°C. Germination (100%) of arthroconidia was observed by 40h. With longer incubation the whole hair follicle was covered by a dense mat of mycelium. Growth was more rapid on the dissected hair follicle which were submerged in

the mycelium earlier as compared to the plucked hair follicles. It was observed that fungal growth was maximal on the shaft of the hair as compared to growth on the other areas. With the plucked hair follicles, in the initial stages, growth of germlings was inhibited on the outer root sheath. It was also noticed that there was lack of fungal growth around the hair bulb which was seen to be swollen (Fig 6:2). Germlings were seen growing along and encircling the hair shaft. The hyphae were seen very prominently on the keratogenous zone. The fungus was seen destroying this zone while the rest of the hair was relatively intact (Fig 6:3).

Longitudinal cuts of dissected hair stained with toluidine blue showed in the controls very clearly the different layers (Fig 6:4). While section of the hair at 24h showed that the arthroconidia had germinated and germlings were growing on the inner root sheaths with evidence of penetration of this layer. At 40h of incubation there was extensive growth of fungal elements on the inner root sheath which was fully invaded by the fungus. The cuticle was not penetrated by the fungus at this stage, though fungal elements were seen entering through distal and proximal ends of the follicle (Fig 6:5). With longer incubations around 4 days, invasion of the shaft was seen from the distal and proximal ends. The inner root sheath was broken up and fully invaded by fungal elements which were seen to penetrate in between the cuticular cells. No invasion of the cortex was observed through the cuticular layers (Fig 6:6). Around 6 days of incubation invasion of the cortex was seen, by fungal elements penetrating through inner root sheath and cuticular cells, and from the distal and proximal ends. Fungal elements were seen growing in the cortex between the fibrils and with time replacing most of the structure (Fig 6:7).

Plucked follicles with outer root sheath dissected off showed abundant growth. Where the outer and inner root sheaths both had been dissected extensive invasion of the follicles was seen with most of the structure replaced by the fungal mycelium (Fig 6:8).

6:5:1:2 Electron microscopy

On scanning electron microscopy control plucked follicles showed a well formed shaft, with its sheaths and the bulb area intact (Fig 6:9) with the cuticular cells being flat and overlapping with the free edge of the cells. Around 24h arthroconidia were seen adhering at the distal end, on the outer root sheath and hair bulb (Fig 6:10). Following inoculation of arthroconidia at the distal end of the follicle, germination of arthroconidia was seen (Fig 6:11) and, germlings of *T. mentagrophytes* begin to grow abundantly along the hair surface, closely encircling the hair shaft towards the proximal end, with no apparent damage to the hair in the early stages of infection. The direction of growth on the hair surface was against the direction in which the cuticular scales overlap (Fig 6:12). In addition some of the hyphae seemed to penetrate beneath the free edge of cuticular cells which in the early stages remain firmly adherent, but were seen to be slightly raised later on. Hair follicles infected with the fungus showed large flaps of cuticle raised from the underlying cortex (Fig 6:13).

Completely invaded hair showed hollowing out of the hair shaft (Fig 6:14) with abundant arthroconidia formation. Arthroconidia were globular with absent rings of fibrillar material normally seen on culture media (Fig 6:15). Around 7 days of incubation most of the hair was covered with mycelium, with the hyphae growing in all directions. Some hair were not visible through the mycelial mat as the growth was so extensive.

Dissected hair follicles showed rapid and extensive growth of fungus and were fully covered with growth by 4 days of incubation (Fig 6:16).

On transmission electron microscopy of plucked hair follicles very close and firm adherence of arthroconidia to the surface of the hair was seen. There was evidence of enzymatic damage taking place allowing the dermatophyte to penetrate the hair (Fig 6:17). In the initial stages separation of the cuticular cells caused by the germlings was seen but without evidence of damage to

these cells. Later on the cuticular cells had become markedly detached from the underlying cortex with germlings penetrating in between them with evidence of damage to the endocuticle of cuticular cells (Fig 6:18). Fungal elements were seen penetrating up to the central part of the hair cortex. Most of the filaments of the mycelium were aligned parallel to the long axis of the cortical cells. Fungal elements were seen intercellularly within the hair (Fig 6:19). No arthroconidia were observed in the cortex.

6:5:2 Inhibitory effect of terbinafine on hair invasion by *T. mentagrophytes*

6:5:2:1 Light microscopy

Toluidine sections of dissected hair follicles in the presence of terbinafine concentration (0.01–1 mg/L) showed growth inhibited though germination of arthroconidia was not inhibited. At concentration of 0.01 and 0.1 mg/L growth was seen on the surface which was much less than control. There was some degree of penetration of the outer cuticular layer but invasion of the cortex was not seen (Fig 6:20). In the bulb area few odd fungal elements were seen on the outside but no frank invasion was seen. At a concentration of 0.01 mg/L fungal elements were observed to be growing in the hair up to the level of the keratinized inner root sheath which was not invaded by the fungus. At a terbinafine concentration of 1 mg/L and greater no growth was observed on the hair follicles with a fungicidal effect of the drug (Fig 6:21).

6:5:2:2 Electron microscopy

Dissected hair follicles treated with terbinafine at a concentration of 1 mg/L resulted in a dramatic inhibition of hyphal growth. Pre-exposure of dissected hair follicles to terbinafine did not prevent adherence or germination

Figure 6:1a

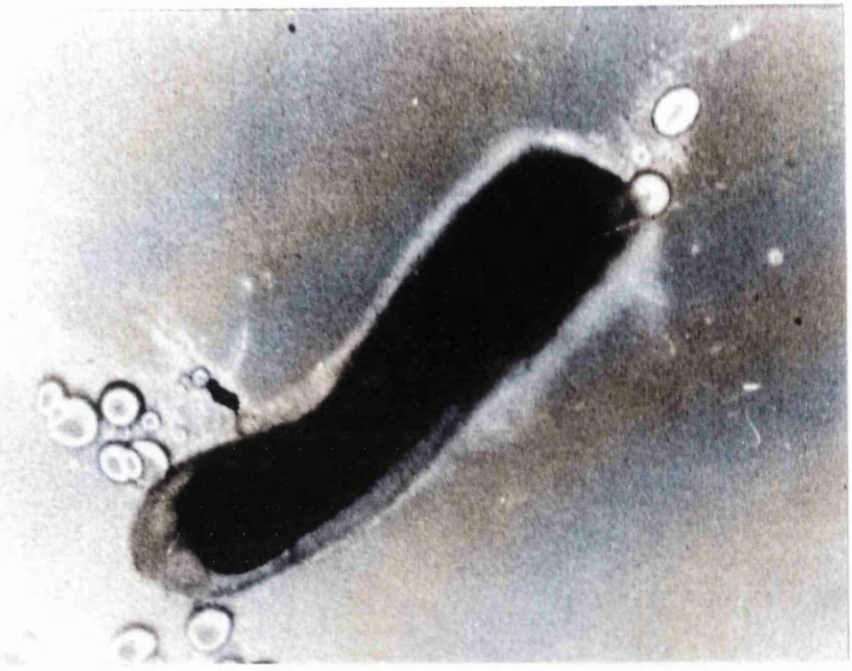


Figure 6:1b

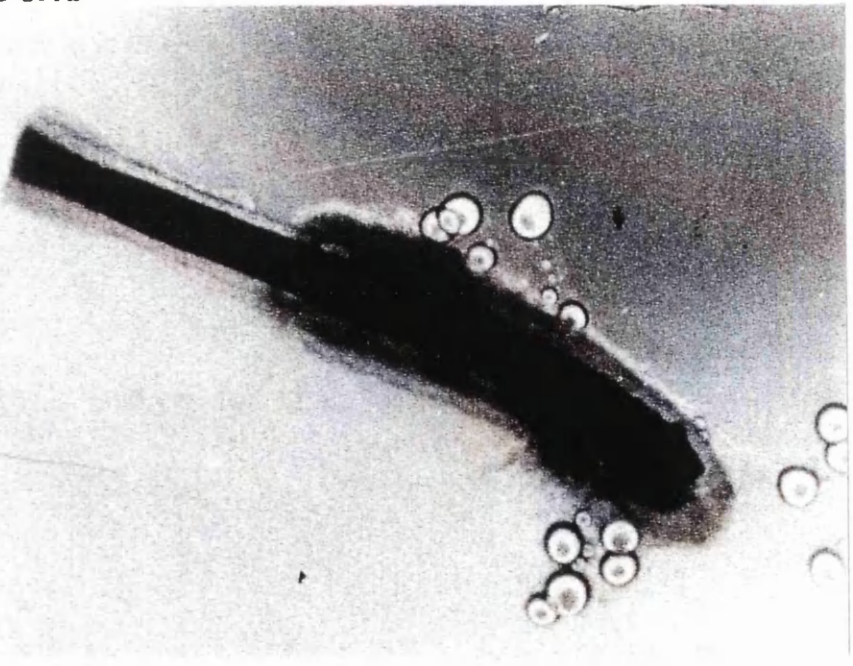


Figure 6:1 Light microscopy appearance of (a) freshly isolated hair follicle and (b) after 96h in culture X 250.

Figure 6:2a

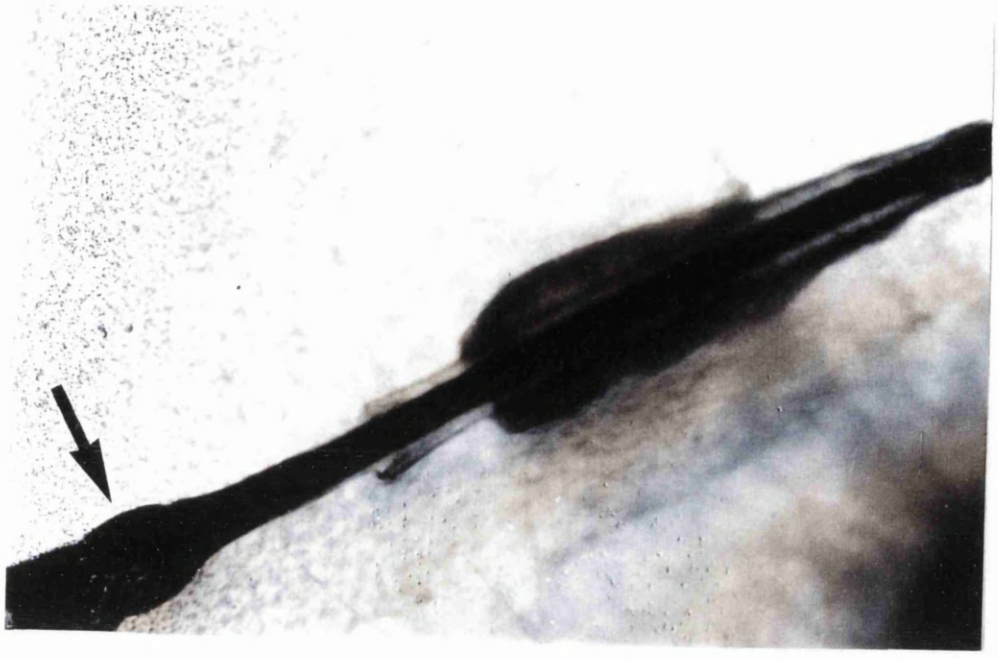


Figure 6:2b

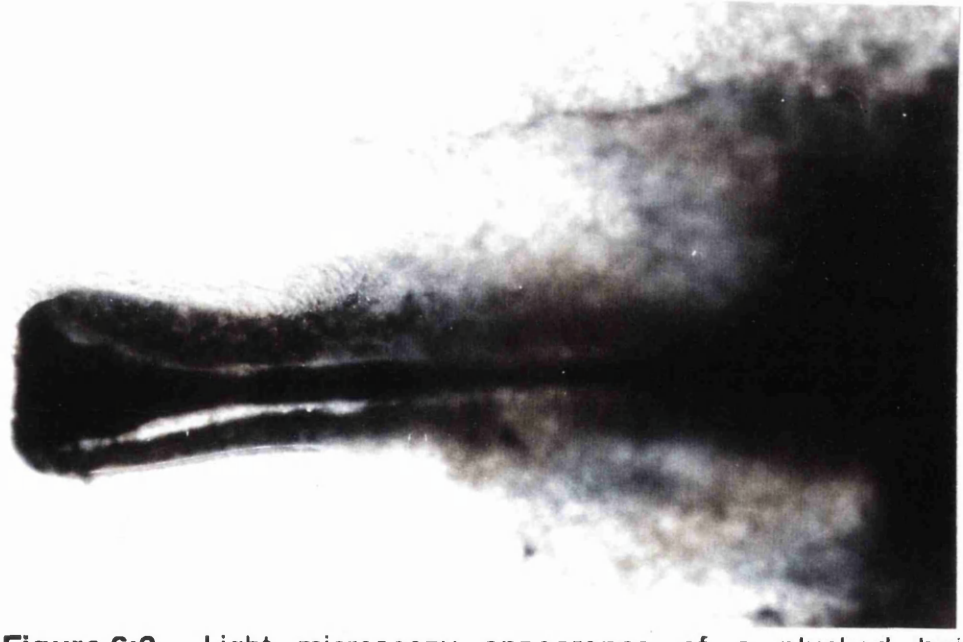


Figure 6:2 Light microscopy appearance of a plucked hair showing (a) the outer and inner root sheath. Mycelial growth is not evident on these layers. Note the swollen hair bulb (arrow) X 250 (b) extensive growth of mycelium. Note the lack of fungal growth around the hair bulb X 400.

Figure 6:3 Light microscopy appearance of a plucked hair showing extensive growth of the mycelium around the keratogenous zone which looks destroyed after 6 days of incubation at 28°C X 400.

Figure 6:4a

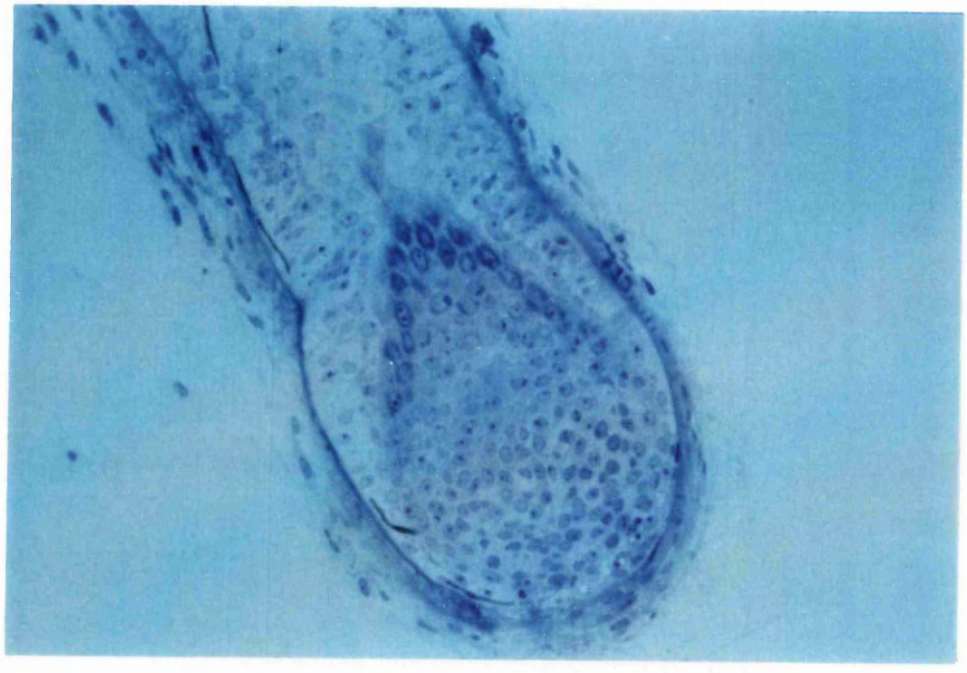


Figure 6:4b

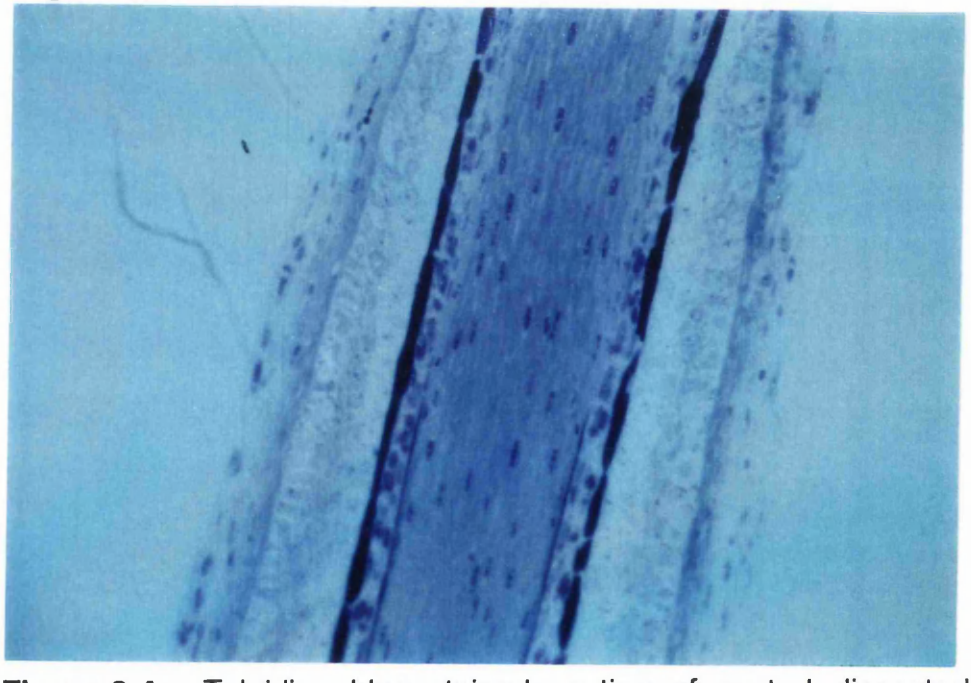


Figure 6:4 Toluidine blue–stained section of control dissected hair follicle showing (a) the well formed bulb (b) the outer and inner root sheaths and hair cortex X 400.

Figure 6:5a

Figure 6:5b

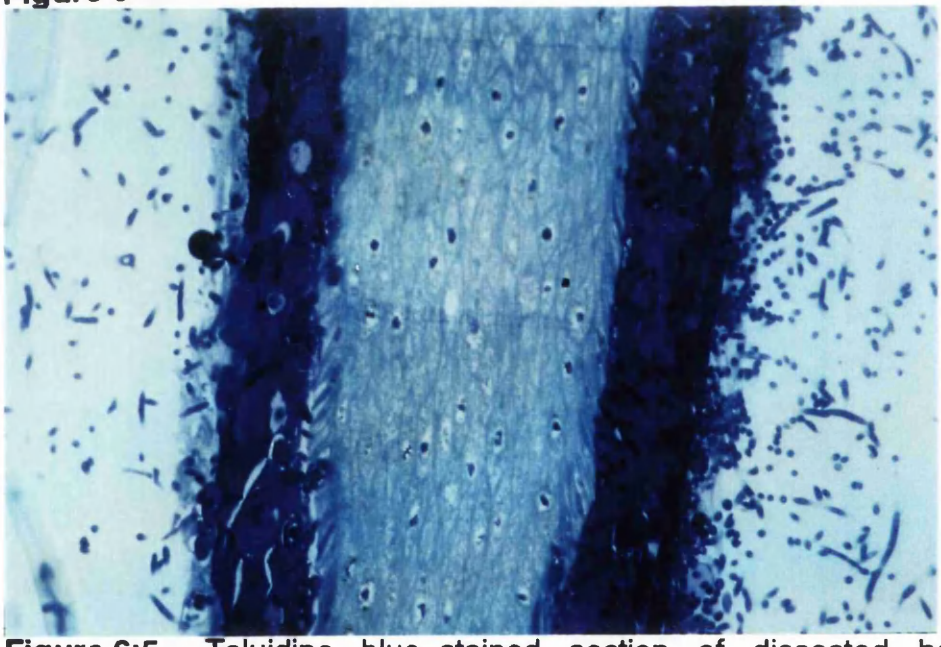


Figure 6:5 Toluidine blue-stained section of dissected hair follicle showing (a) extensive growth of fungus in the inner root sheath. At the level of the cuticular cells there is evidence of inhibition of fungal invasion of the hair cortex X 400 (b) invasion of the follicle from the proximal end X 250. 40h of incubation at 28°C.

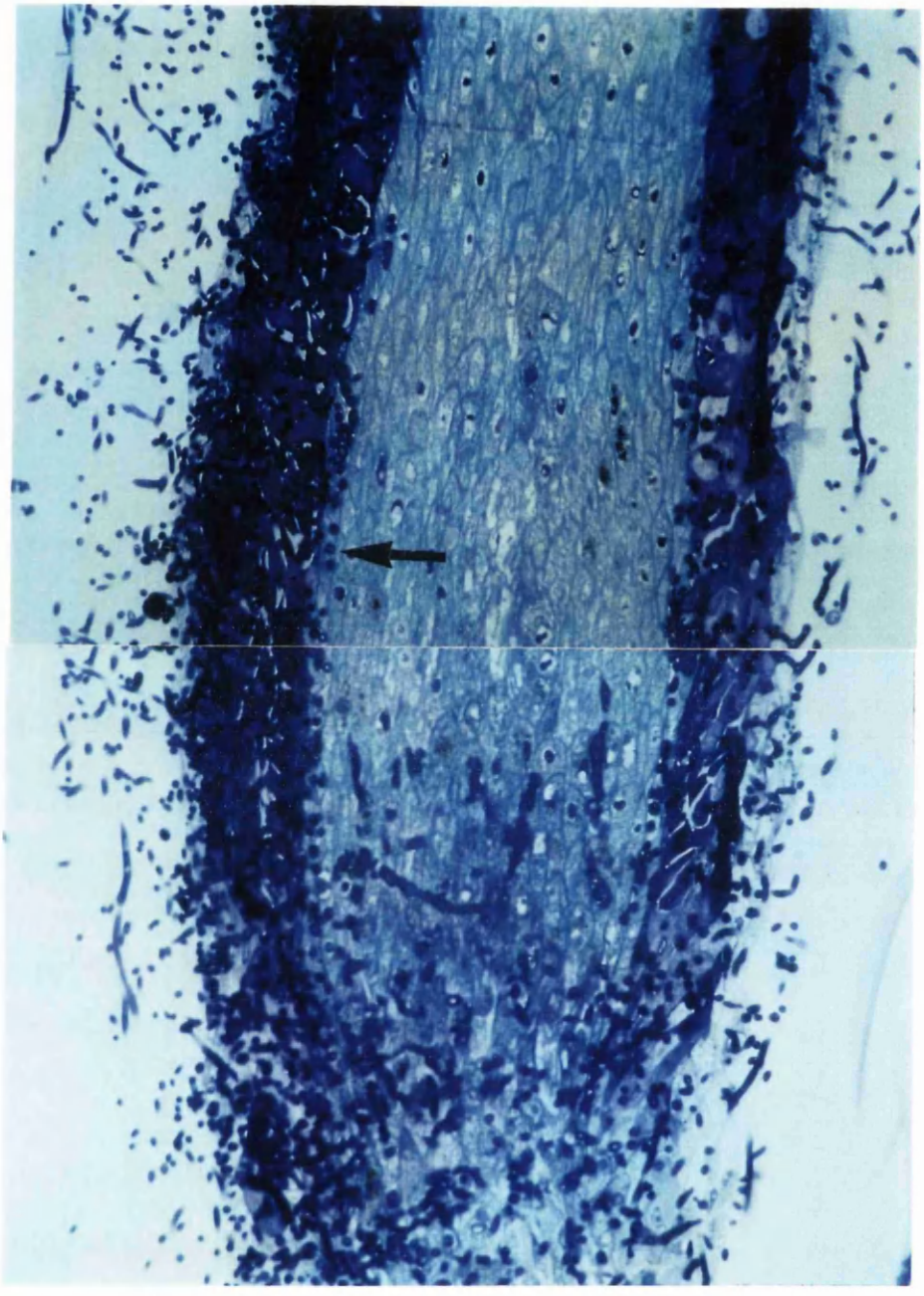


Figure 6:6 Toluidine blue-stained section of dissected hair showing extensive growth of fungus in the inner root sheath. At the level of the cuticular cells there is evidence of inhibition of fungal invasion of the hair cortex, although a few fungal elements are seen within the cuticle (arrow) Fungal elements are seen entering the cortex from the proximal end. 4 days of incubation at 28°C X 400.

Figure 6:7a

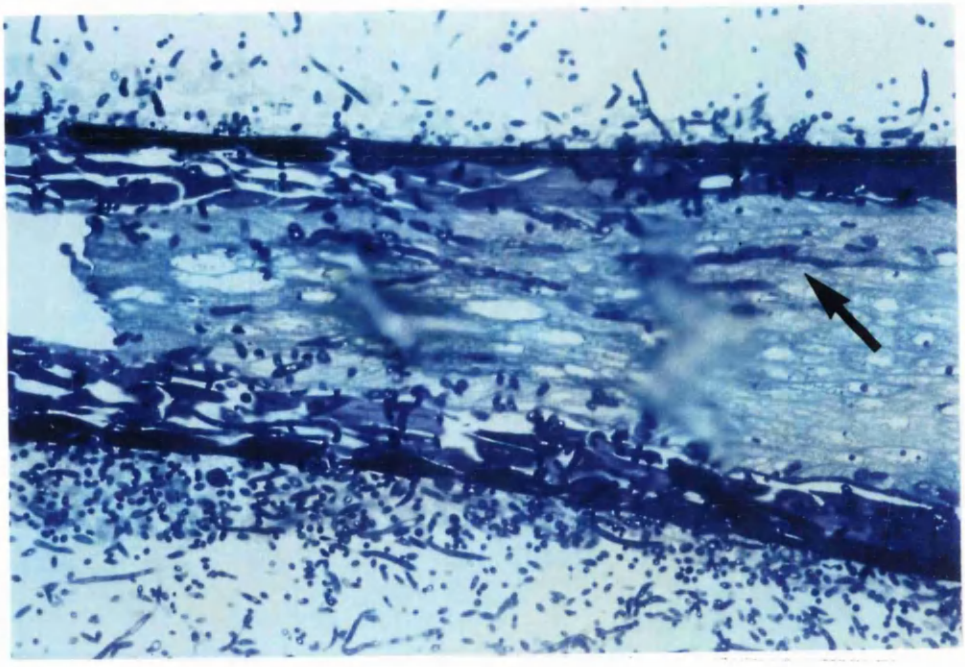


Figure 6:7b

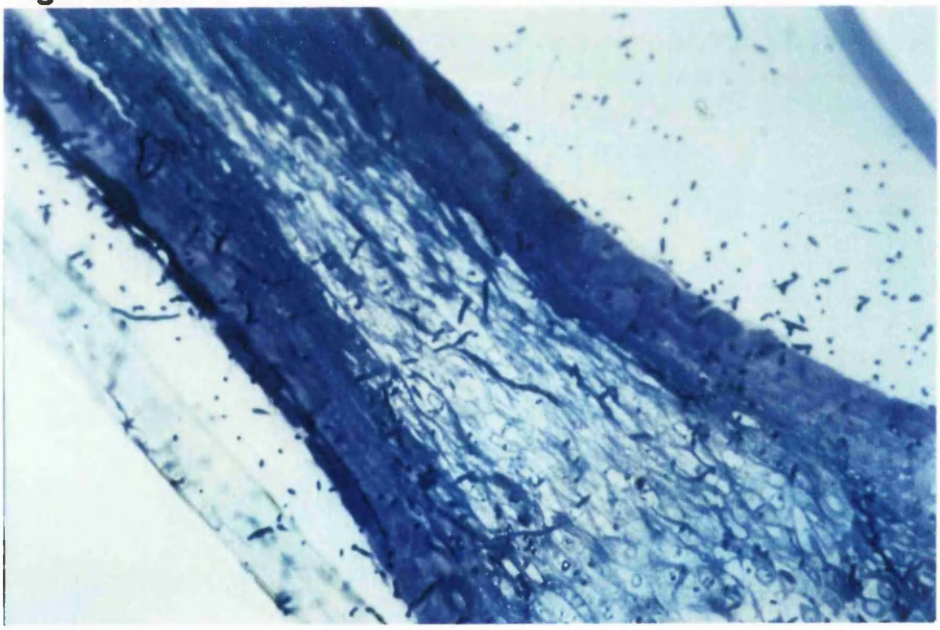


Figure 6:7 Toluidine-blue stained section of dissected hair showing extensive growth of fungus (a) Note the fungal elements breaking up the inner root sheath and growing within the hair cortex (arrow) X 400 (b) the hair fibrils are separated and fungal elements are growing from the proximal end of the follicle towards the distal end X400 (c) extensive growth of the fungus with most of the sheaths having been digested X 250. 6 days of incubation at 28°C.

Figure 6:7c

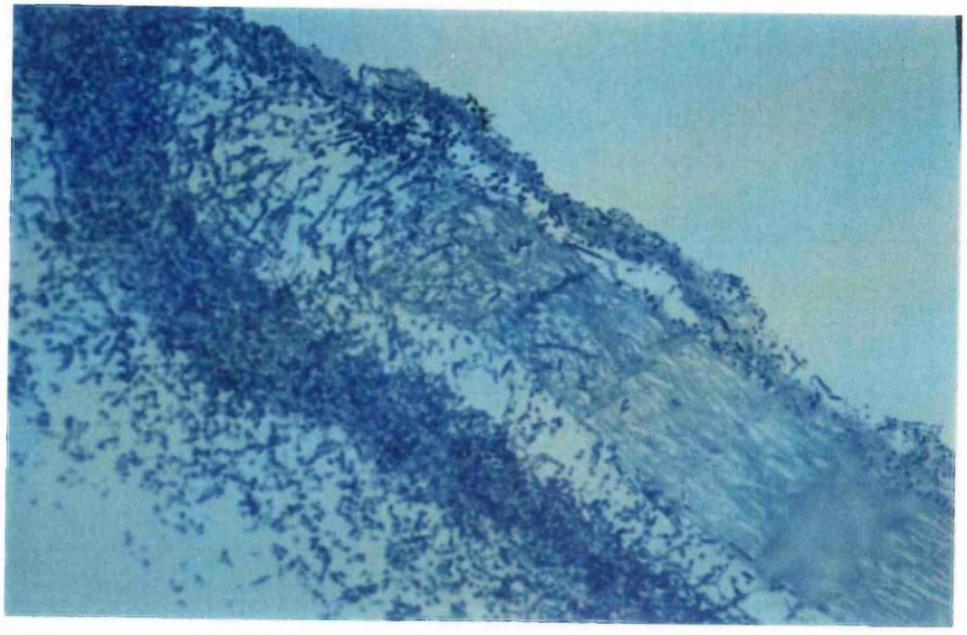


Figure 6:8a

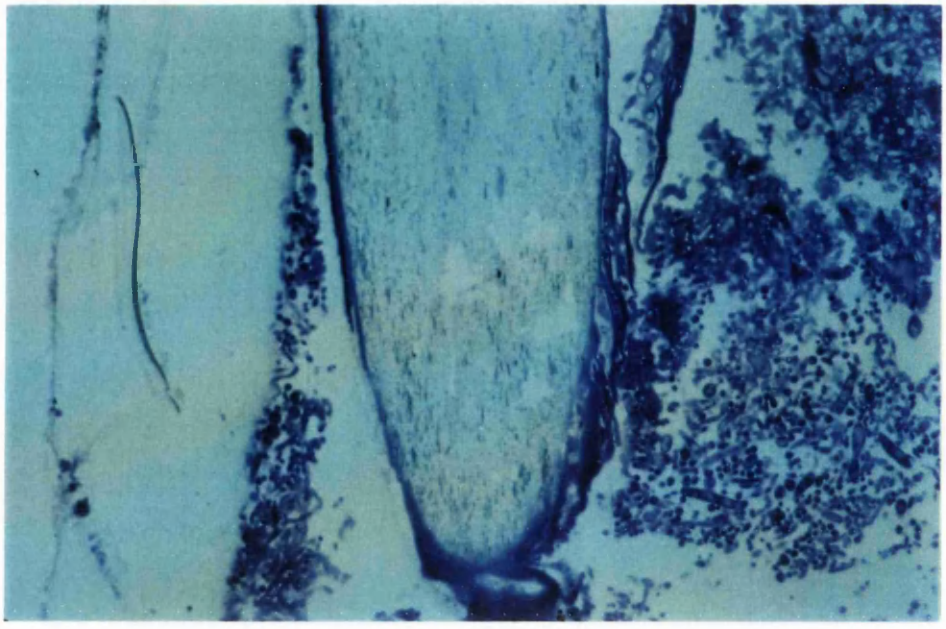


Figure 6:8b

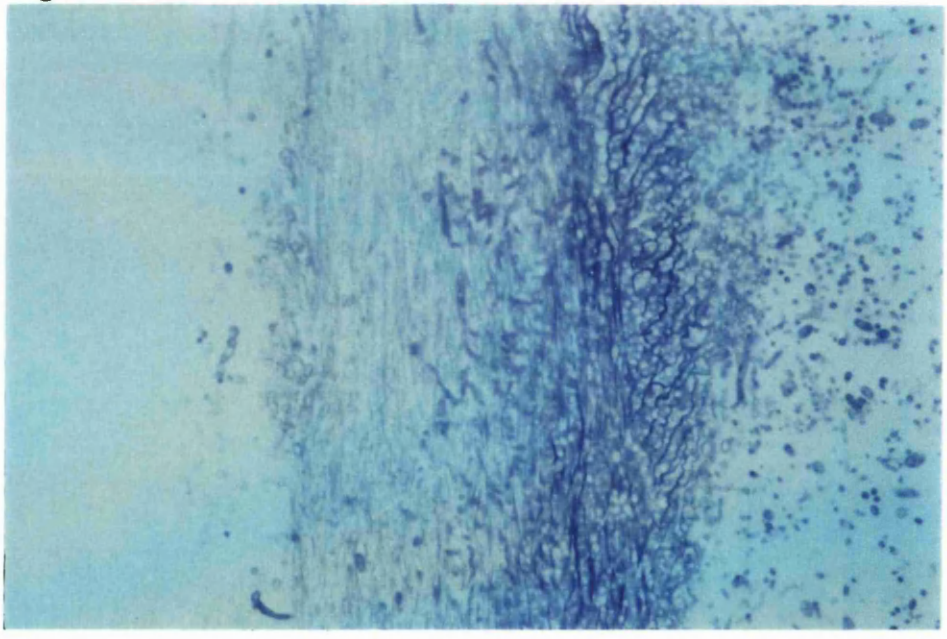


Figure 6:8 Toluidine blue-stained section of plucked follicles showing (a) outer root sheath dissected off and abundant fungal growth (b) outer and inner root sheath both dissected off and extensive invasion of the follicles by fungal mycelium. 6 days of incubation at 28°C X 400.

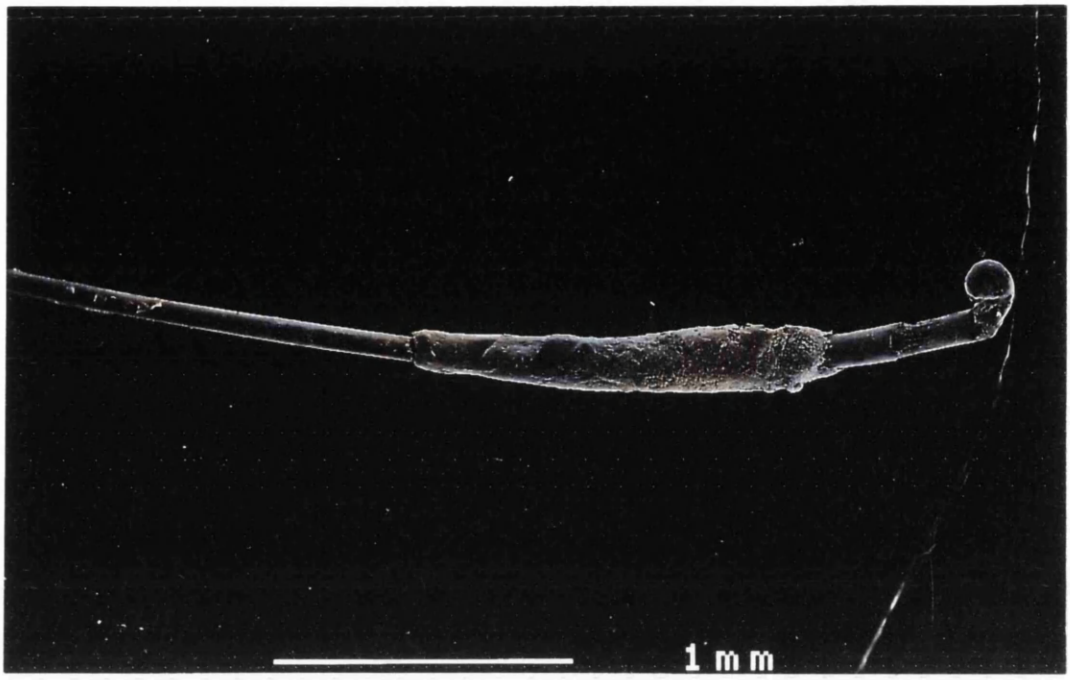


Figure 6:9 Scanning electron micrograph of a plucked hair showing a well formed shaft with its sheaths and an intact bulb area X 66.

Figure 6:10a

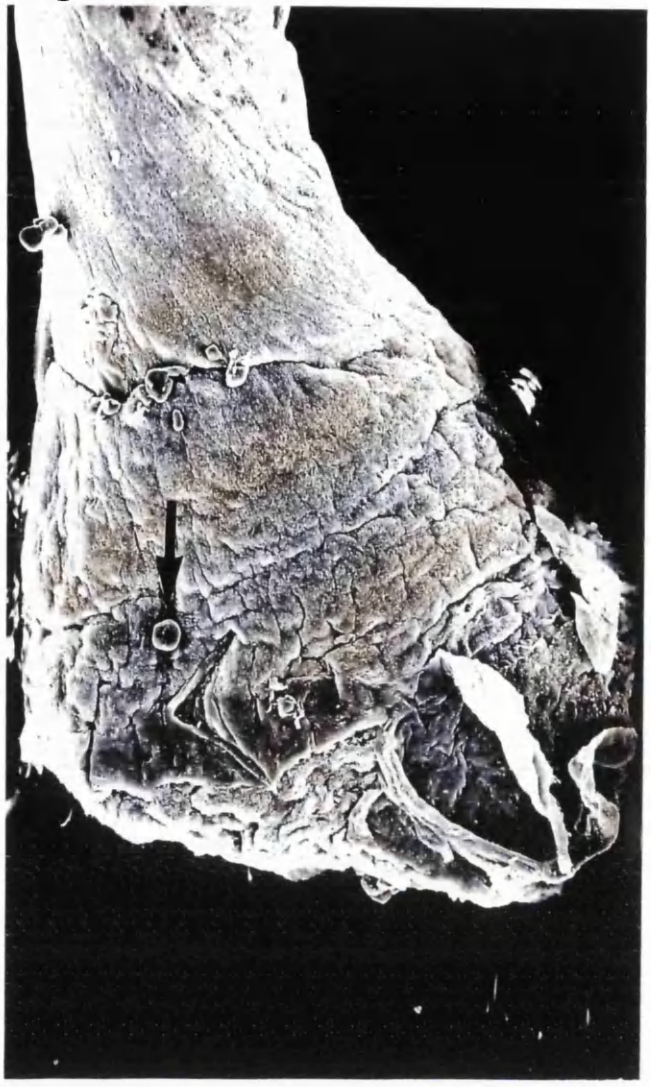


Figure 6:10b

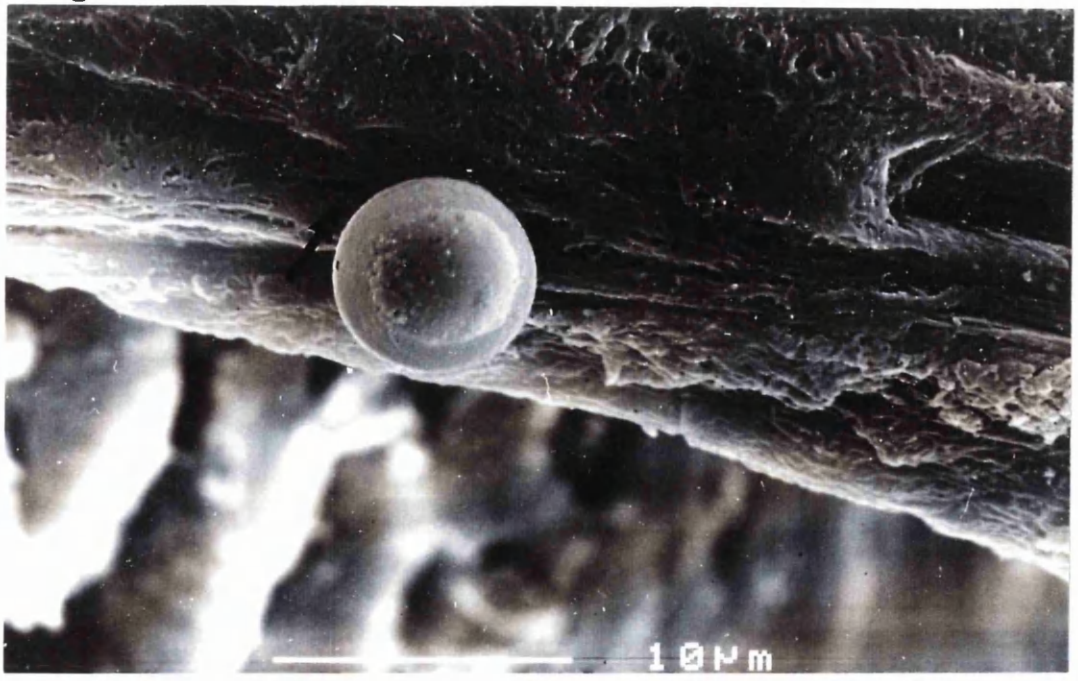


Figure 6:10 Scanning electron micrograph of a plucked hair follicle showing (a) adherence of ungerminated arthroconidia to the bulb area (arrow) X 800 (b) adherence of an arthroconidium to the outer root sheath. Note the depression formed (arrow) where the arthroconidium is attaching X 6600.

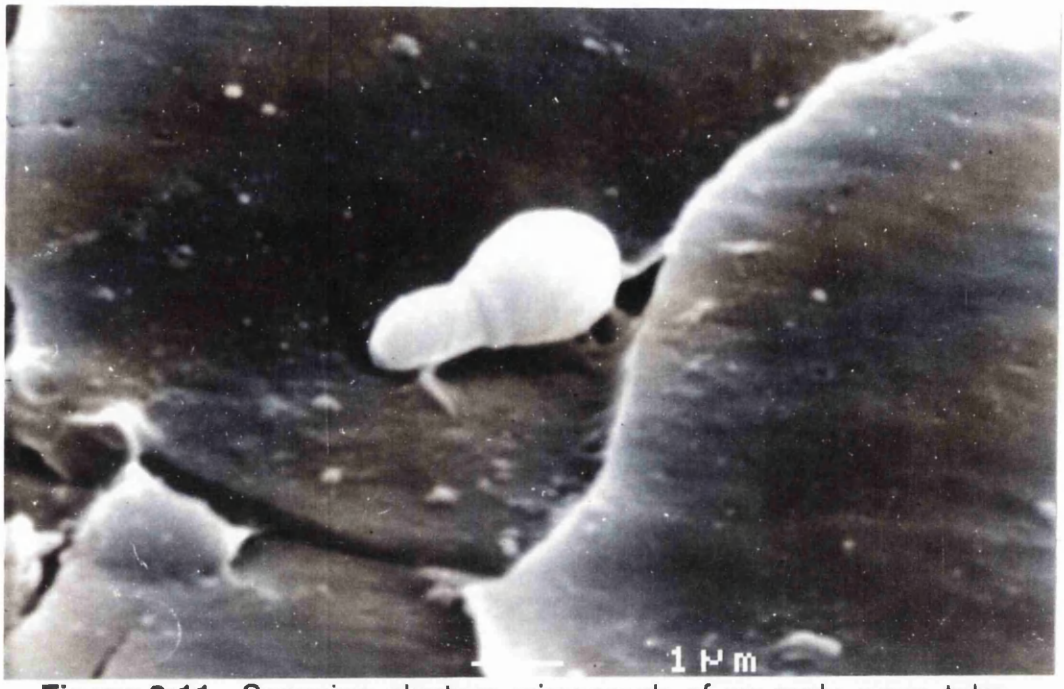


Figure 6:11 Scanning electron micrograph of an early germ tube lying on the cuticular scales of a plucked hair follicle. Note the germ tube emerging after 16h incubation at 28°C X 20000.

Figure 6:12a

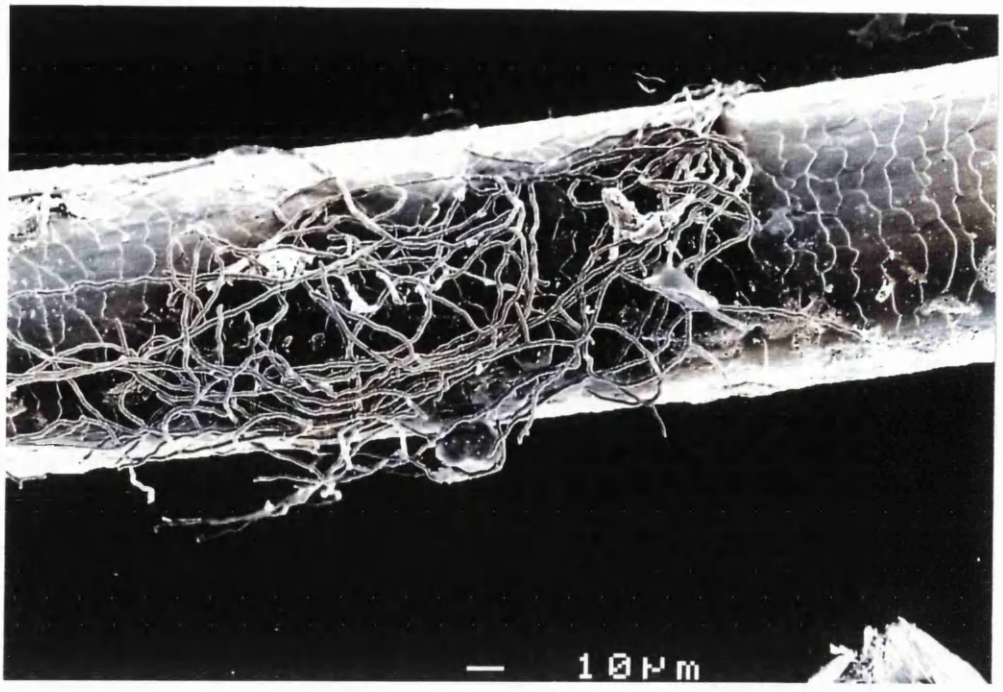


Figure 6:12b

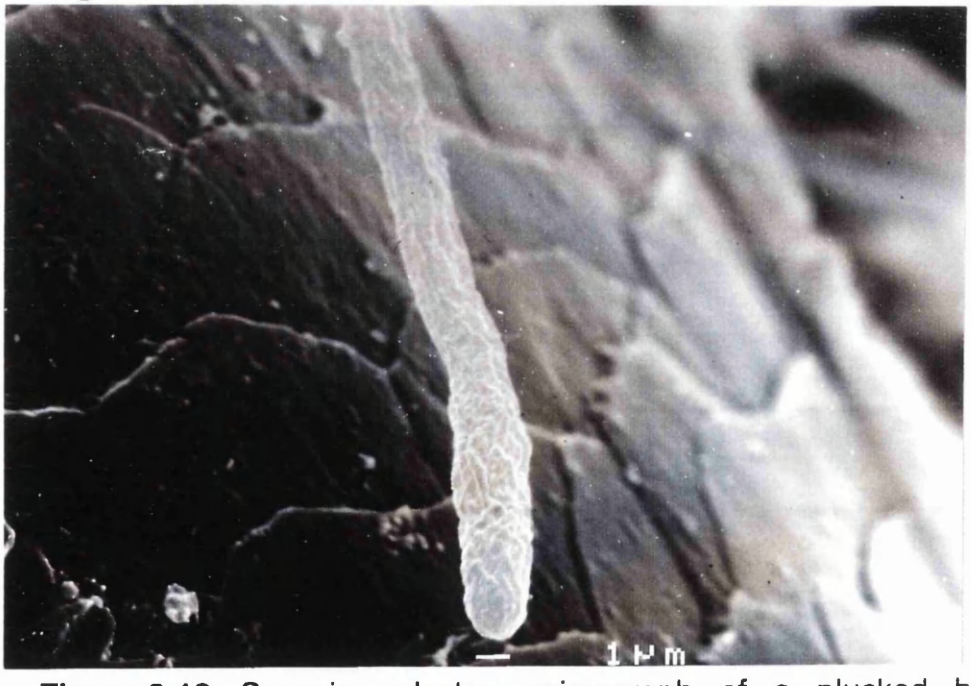


Figure 6:12 Scanning electron micrograph of a plucked hair showing (a) growth of fungal hyphae, encircling the hair shaft X 800 (b) the tip of the hyphae is growing on the outer surface of a hair shaft in the opposite direction to which the cuticular cells overlap X 8000.

Figure 6:13a

Figure 6:13b

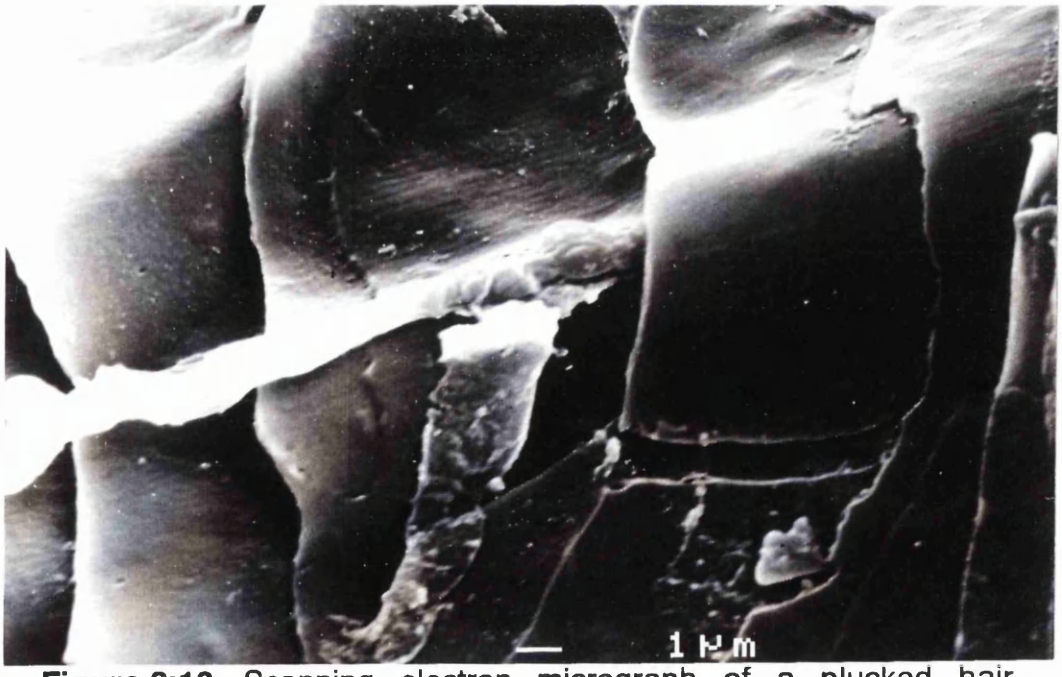


Figure 6:13 Scanning electron micrograph of a plucked hair infected with the fungus (a) Note the flaps of cuticle raised (arrow) X 700 (b) a hyphae penetrating between cuticular cells X 10000.

Figure 6:14a

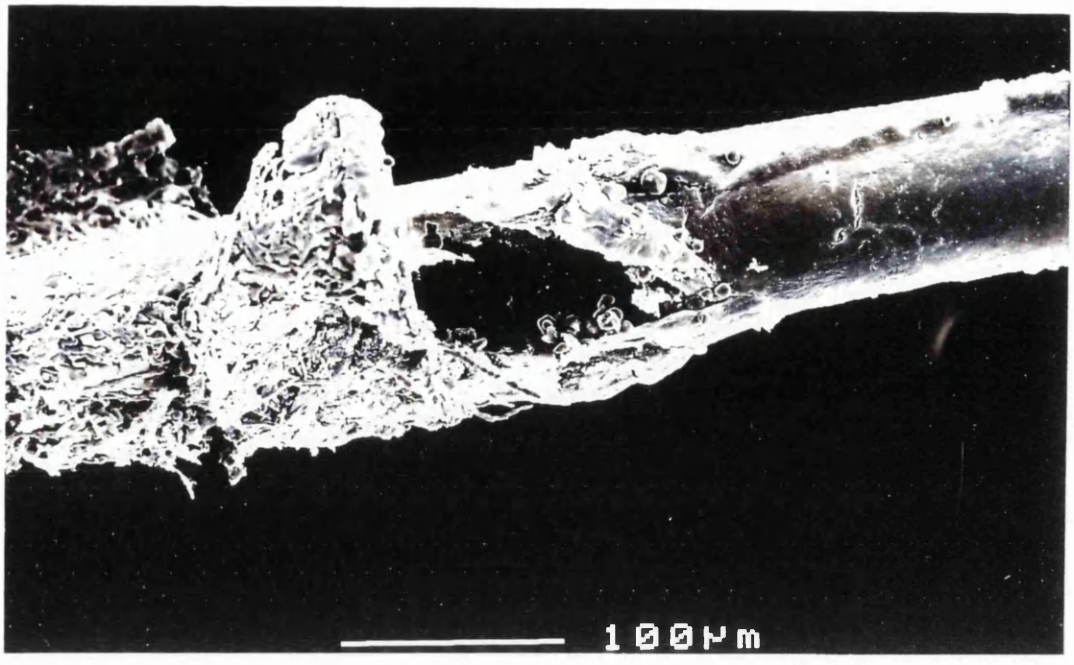


Figure 6:14b

Figure 6:14 Scanning electron micrograph of a plucked hair infected with the fungus (a) Note the cavity formed in the hair shaft X 500 (b) the cuticular scales of the hair can be seen through the cavity. There are arthroconidia adhering to the edges of the cavity (arrow) X 1600.

Figure 6:15 Scanning electron micrograph of arthroconidial formation on the hair. Note the globular appearance of the arthroconidia (arrow) X4000.

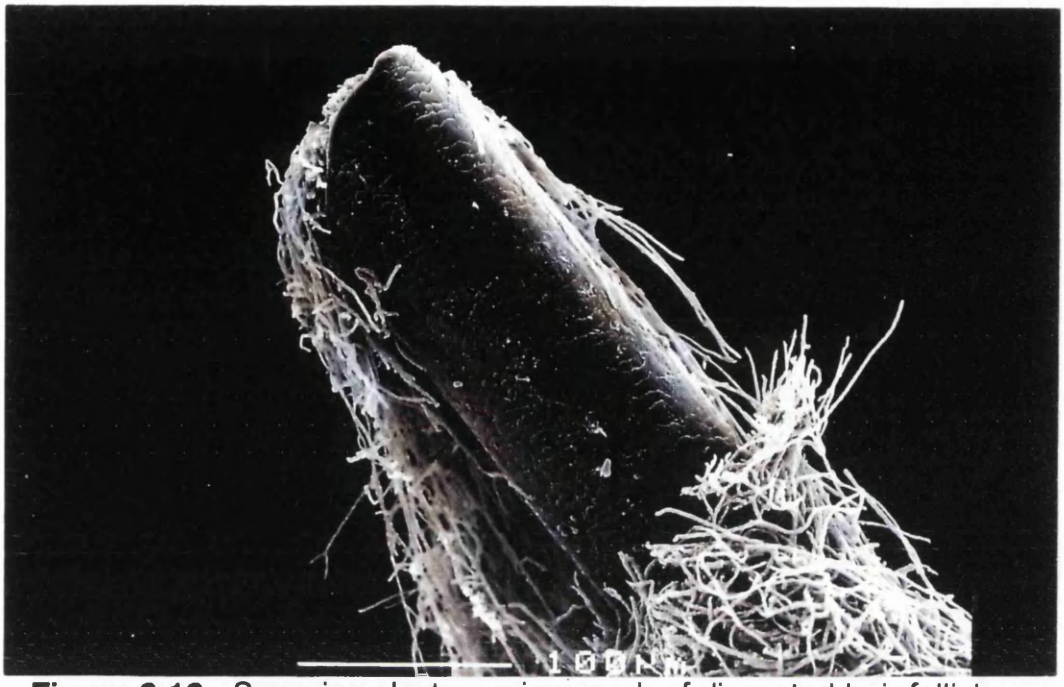


Figure 6:16 Scanning electron micrograph of dissected hair follicle showing extensive growth of the fungus after 4 days of incubation at 28°C X 540.

Figure 6:17a

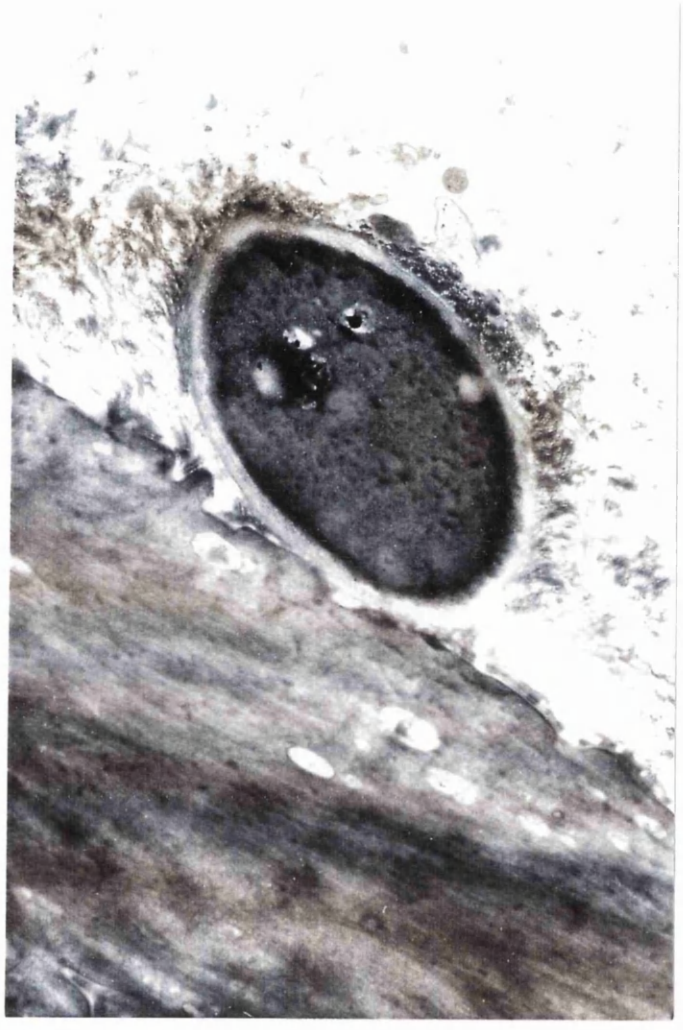


Figure 6:17 Transmission electron micrograph showing adherence and invasion of the hair by arthroconidia (a) Note the close adherence with evidence of enzymatic damage X 18000 (b) firm adherence and penetration is seen taking place X 15000 (c) invasion of the surface, is taking place X 22500.

Figure 6:17b

Figure 6:17c

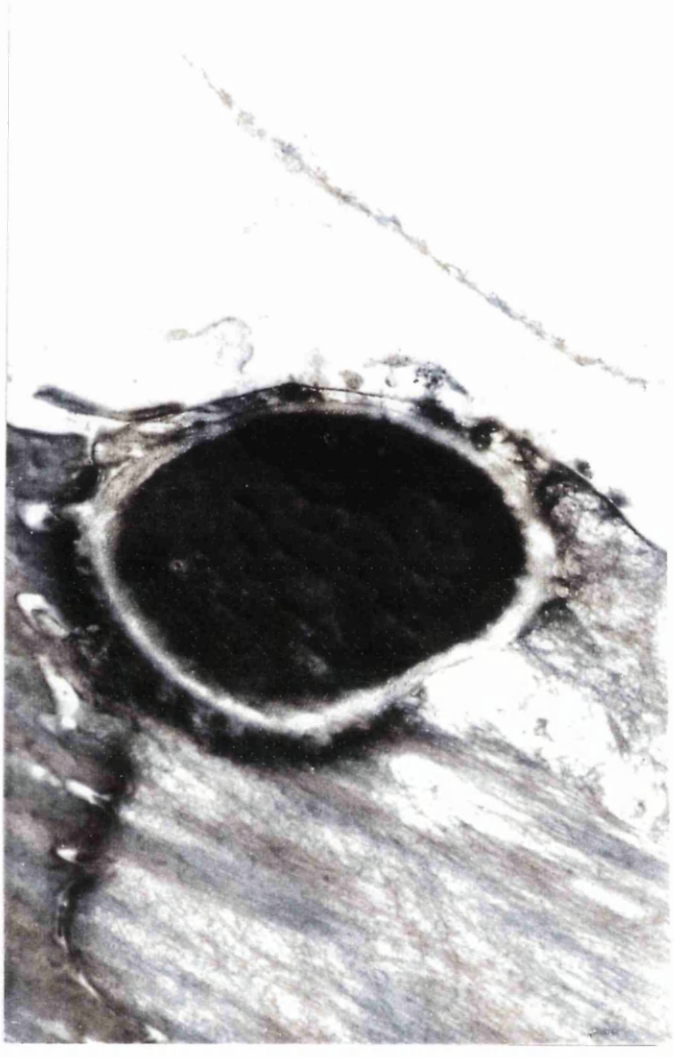


Figure 6:18a

Figure 6:18b

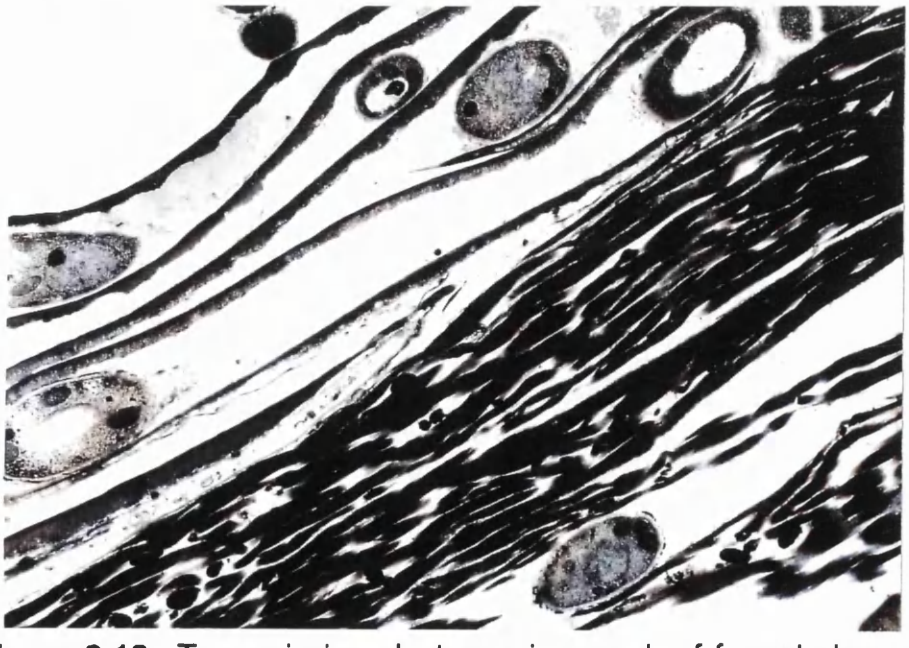
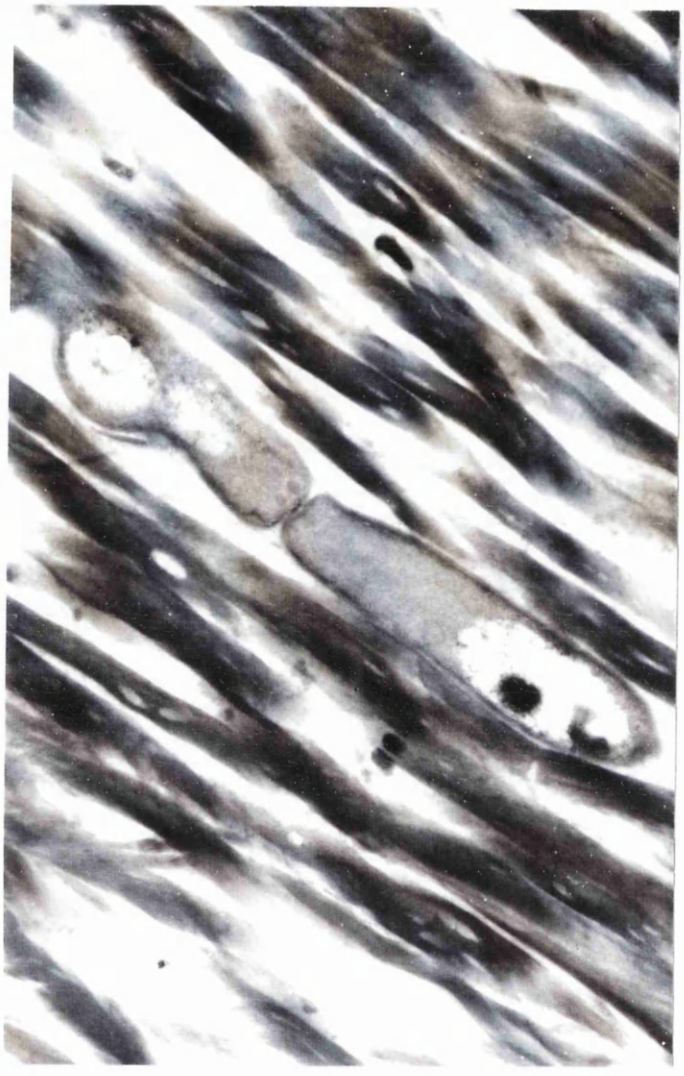


Figure 6:18 Transmission electron micrograph of fungal elements (a) penetrating in between and separating layers of the cuticular cells. Note the appearance of slight damage to the endocuticle (arrow) X 15000. (b) marked detachment of the cuticular cells from the underlying cortex with fungal elements penetrating in between them X 10000.

Figure 6:19a

Figure 6:19 Transmission electron micrograph of fungal elements penetrating a hair (a) Note the fungal hyphae growing on the cuticle, between the cuticular layers and the cortex (arrow), and within the hair cortex X 6000 (b) fungal elements penetrating between the cortical cells and lying parallel to the cells in the intercellular space. No evidence of intracellular penetration can be seen X 15000.

Figure 6:19b



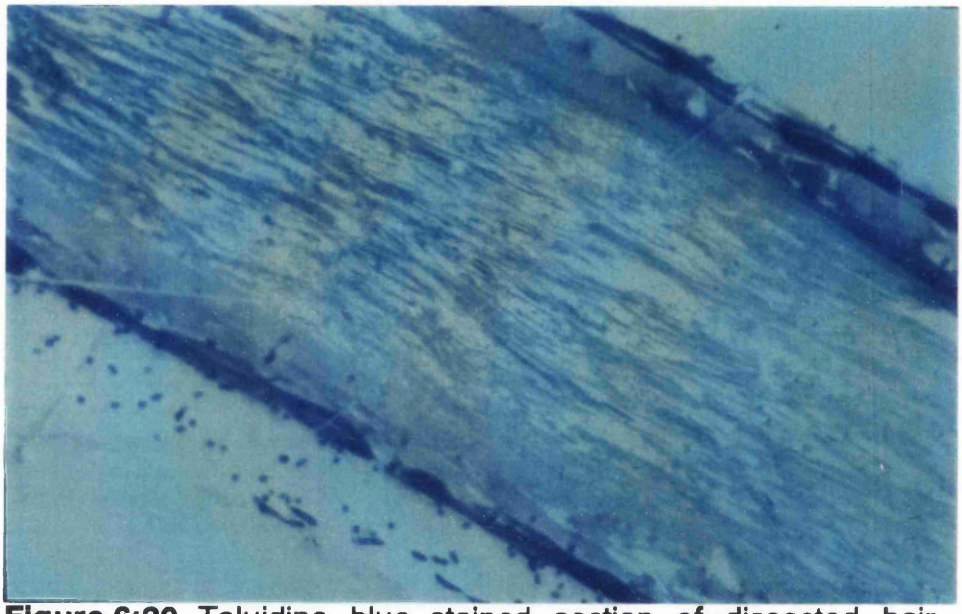


Figure 6:20 Toluidine blue–stained section of dissected hair follicle showing growth inhibition following exposure to 0.1 mg/L terbinafine. There is very slight growth on the follicle after 4 days of incubation at 28°C X 400.

Figure 6:21a

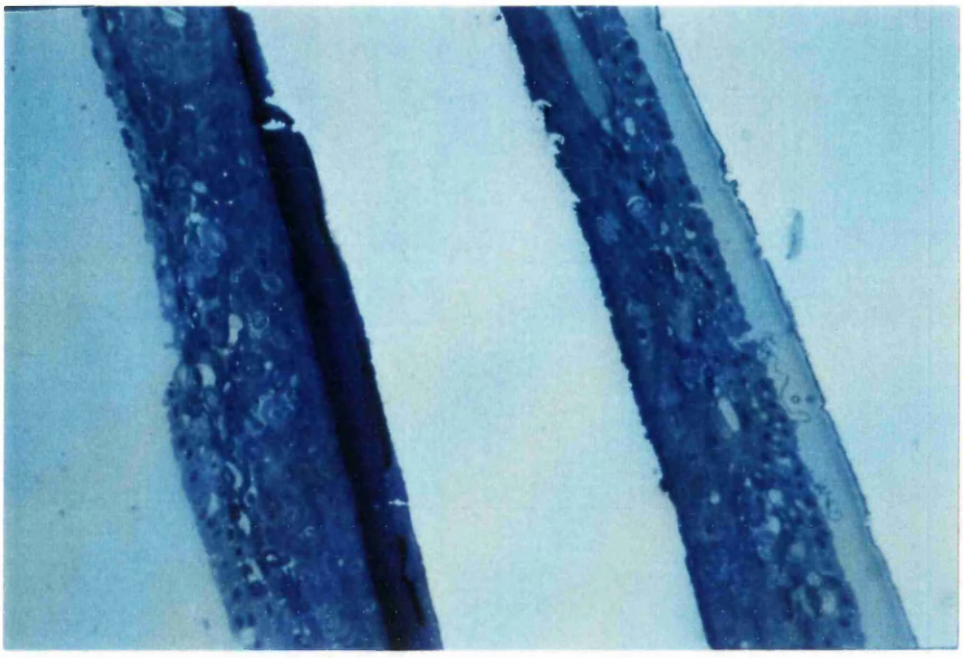


Figure 6:21b

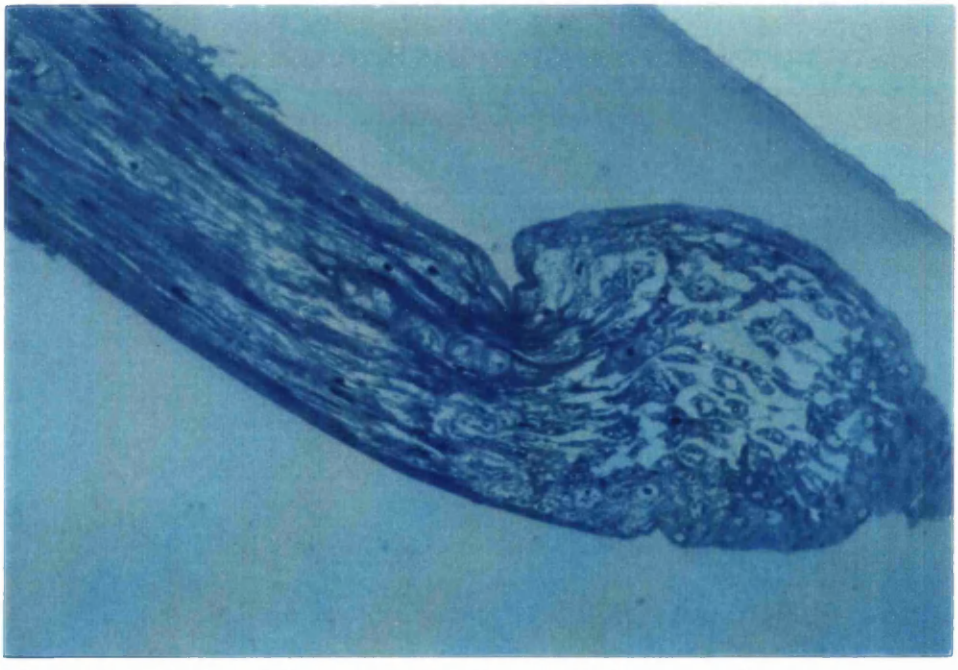


Figure 6:21 Toluidine blue–stained section of dissected hair follicles showing absence of fungal growth after exposure to 1 mg/L of terbinafine after 4 days of incubation at 28°C (a), (b) X 400.

Figure 6:22a

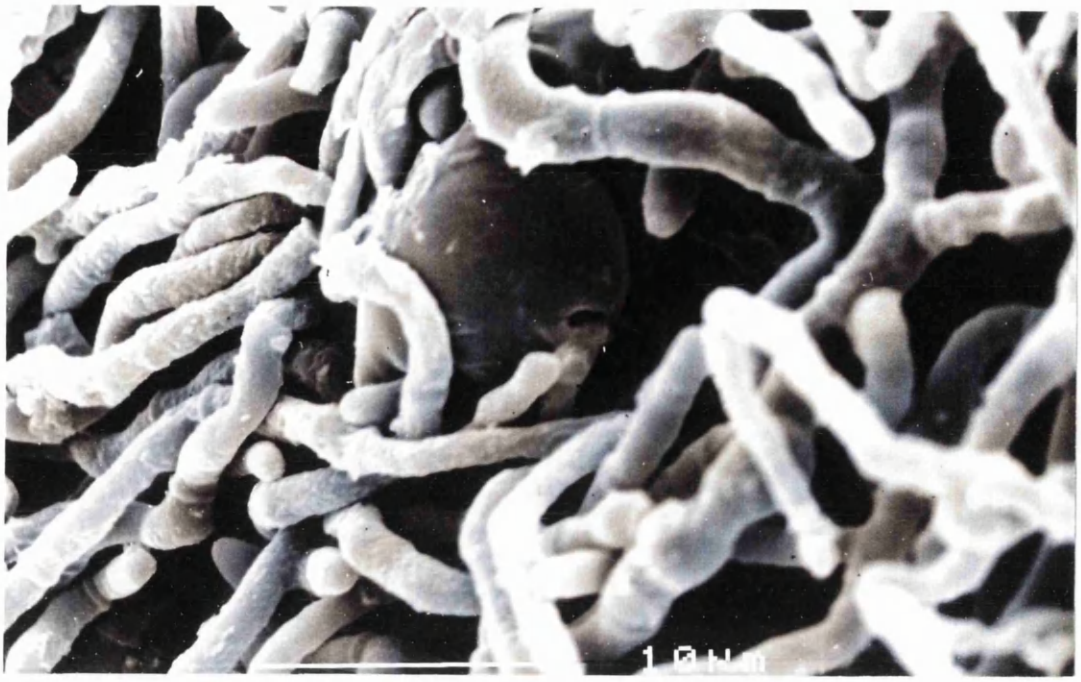


Figure 6:22b

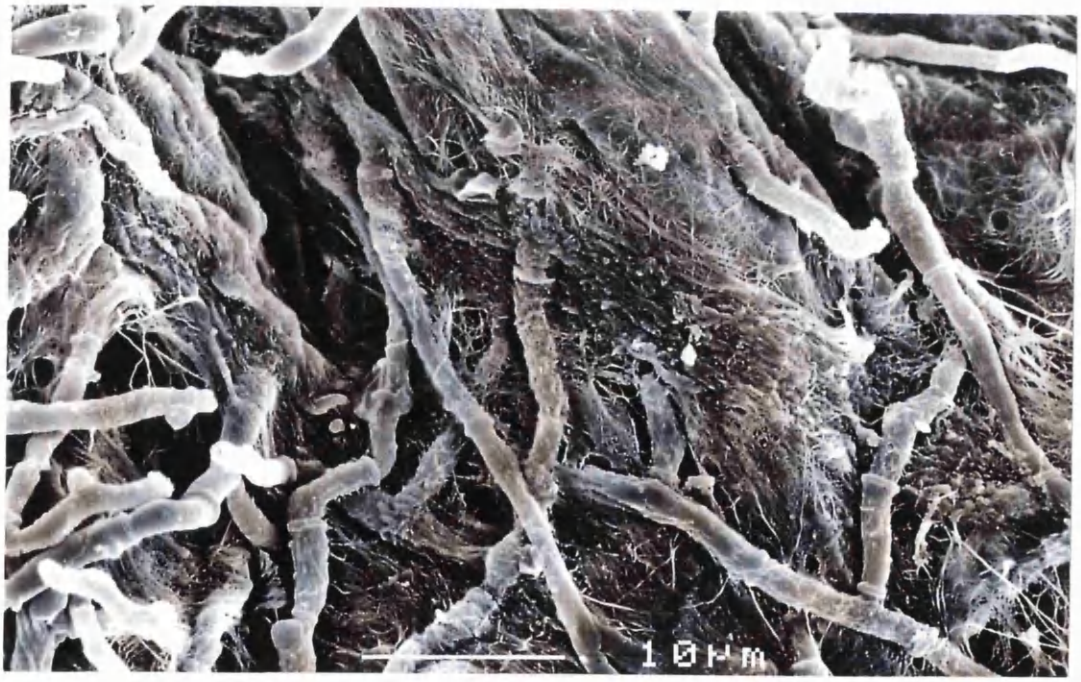


Figure 6:22 Scanning electron micrograph of dissected hair follicles which was exposed to 0.1 mg/L terbinafine. Note the rough and wrinkled surface of the hyphae X 7000 (b) invasion of the hair follicle is seen by hyphae, the surface of which are showing exfoliation X 4400.

Figure 6:23a

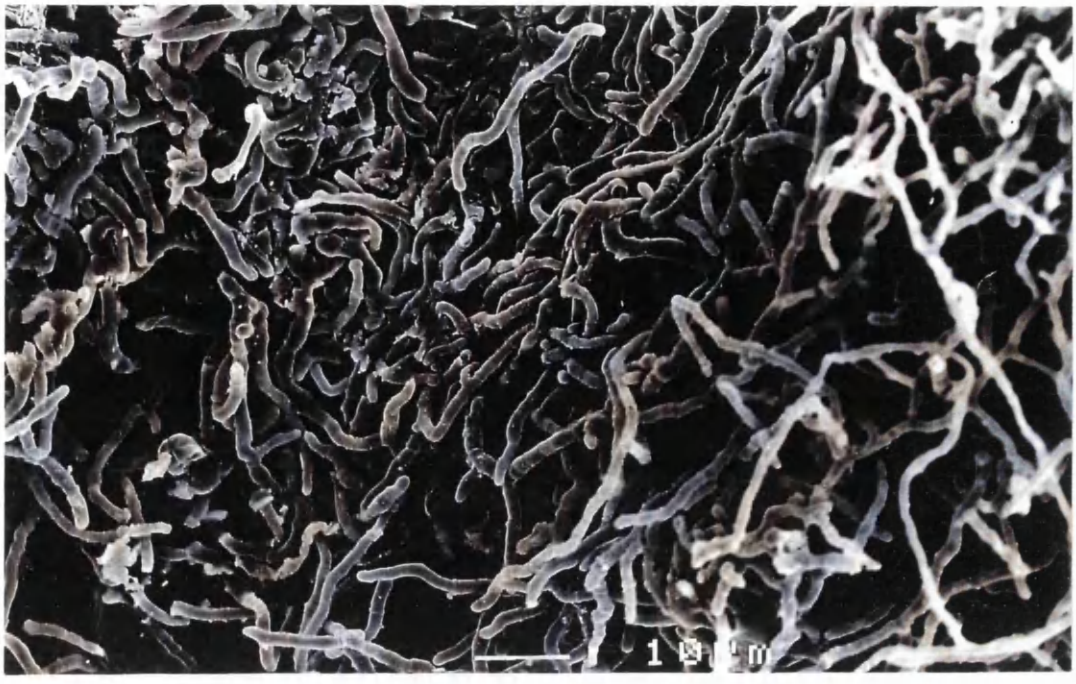


Figure 6:23b

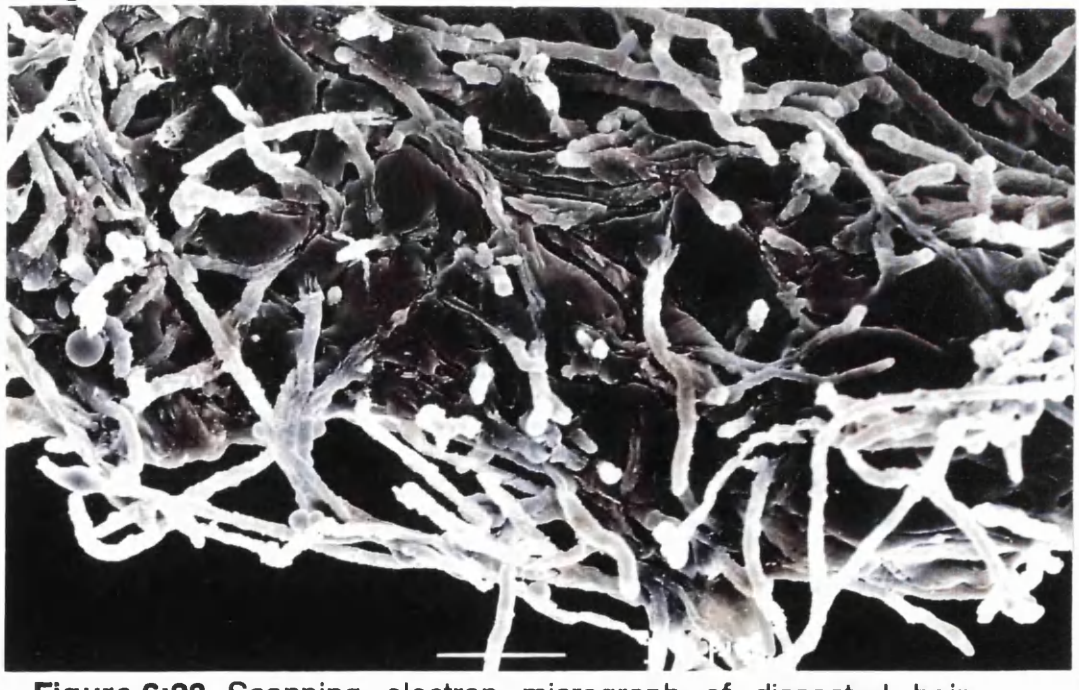


Figure 6:23 Scanning electron micrograph of dissected hair follicles with fungal hyphae exposed to 0.01 mg/L of terbinafine (a) Note the matted hyphae X 2000 (b) and areas of sparing on the follicle surface X 1400.

Figure 6:24a

Figure 6:24b

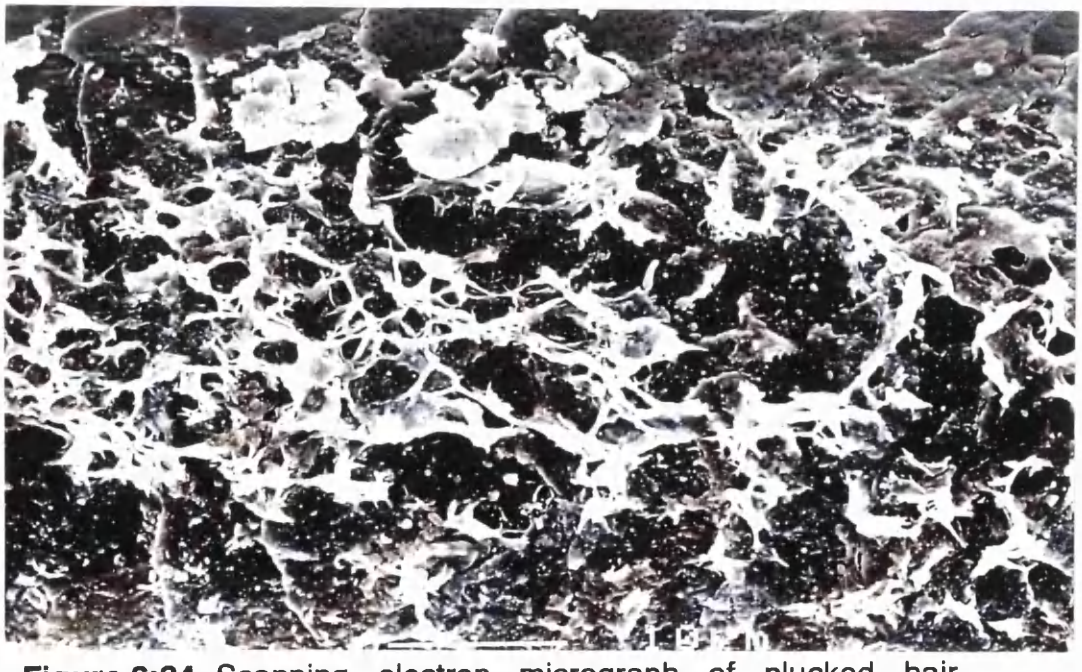


Figure 6:24 Scanning electron micrograph of plucked hair follicles pre-exposed to 1 mg/L terbinafine (a) Note the damaged arthroconidia with a large pore (arrow) X 5000 (b) remnants of a mycelium lying on the hair surface X 3500.

of arthroconidia. At a concentration of 0.1 mg/L arthroconidia were seen to have a rough surface with pores in evidence. Some of the hyphae showed exfoliation of the surface layer, with rough and wrinkled surfaces (Fig 6:22).

Invasion of hair follicles was seen when terbinafine was tested at concentrations of 0.1 mg/L and below. At concentrations of 0.01 and 0.1 mg/L hyphae were matted together with areas of sparing on the hair follicle (Fig 6:23). At concentration of 1 mg/L and above terbinafine was observed to be fungicidal.

Plucked hair follicles pre-exposed to a terbinafine concentration of 1 mg/L resulted in a fungicidal effect with only collapsed hyphal remnants of a mycelium and damaged arthroconidia (Fig 6:24).

6:6 Discussion

6:6:1 A novel *in vitro* hair model for the study of hair invasion by dermatophyte fungi

In this study hair invasion was observed in terminal hair follicles isolated by micro-dissection and maintained in tissue culture in the absence of serum. There was luxuriant growth of the mycelium on the hair follicle and the area around it.

The conventional methods available for the isolation of intact hair follicles from humans are either to pluck hair from the skin using mechanical force, or to microdissect follicles from biopsy tissues. Hair follicles can also be isolated by shearing, a technique that recovers the whole follicle intact and with no evidence of tissue damage. Although the follicles remain metabolically viable for several days in culture, they do not grow to produce a new hair (Green *et al.*, 1986). Bassukas and Hornstein (1989) have shown that plucked follicles may be damaged by the tearing force exerted to remove them. In contrast, by careful micro-dissection it is possible to obtain undamaged human hair follicles

which grow *in vitro* at an *in vivo* rate. Philpott *et al.* (1991) have shown that it is possible to maintain isolated human hair follicles in serum-free medium *in vitro* for up to 10 days during which time the dermal papilla remains elongated, and more significantly the hair follicles continue to produce a keratinized hair shaft with the hair root sheaths growing with the hair shafts. Hair follicles used in the present study showed an increase in their length of the keratinized shaft indicating that they were viable.

Observations of freshly isolated human hair follicles made at 24h intervals show that *in vitro* the follicles increase in length over 4 days in culture. The increase in length was not associated with any disruption of hair follicle architecture and the length increase was attributed to the production of a keratinized hair shaft. It has also been shown that the pattern of keratin synthesis remained unchanged in hair follicles maintained for 4 days (Philpott *et al.*, 1990). These studies indicate that human hair follicles maintained in culture do not apparently have a requirement for serum for their elongation and the serum factors may in fact be inhibitory. Recently, Imai *et al.* (1993) successfully cultured hair follicles in serum-free medium and showed histologically that the hair bulb and germinative cells maintained their normal morphology for 96h in culture. Dissected hair follicles used in this study were inoculated with arthroconidia on the 4th day when a reasonable hair shaft had appeared.

The advantages of using dissected hair follicles is that one can see the most proximal part of the hair with its intact sheaths and also study the effect of the connective tissue sheaths on the growth of the fungus. The extension of exposed outer root sheath on new shaft may effect the fungus. In clipped hair one only looks at the dead, keratinized hair shaft after emergence from the skin, and the growth of the fungus on the viable portion of the hair cannot be studied. While in plucked hair the most proximal part (root), or the emergent hair shaft can be used to study the growth of dermatophyte fungi.

There were similarities between the findings in this study of the process of hair invasion by dermatophyte fungi and that in the natural disease and as

the experimental system described here with dissected hair is very close to the *in vivo* situation therefore hair roots cultured in this way can serve as a useful model for studying the morphological transition of arthroconidia and for the detailed study of the process of hair invasion by different dermatophyte fungi.

6:6:2 *In vitro* hair invasion by *T. mentagrophytes*

The results show that arthroconidia inoculated on dissected and plucked hair germinate rapidly. The arthroconidia and germlings have an affinity to grow on and around the follicle. Growth was observed to begin at the shaft end and extend along the hair shaft towards the bulb area.

It is known that dermatophytes are keratinophilic fungi and the ability to invade hair *in vitro* is a property of the keratinophilic fungi in general. Vanbreusenghem (1952) described that the majority of dermatophytes are able to grow on hair and invade it, though the growth of dermatophytes on hair *in vitro* was quite different from that *in vivo*. Differences in the structure of the keratinaceous tissue is possibly a reason for the irregular growth of dermatophytes in the skin, nail and hair. One of the properties of dermatophytes is their ability to decompose keratin. The mechanism of dermatophyte decomposition of hair keratin is not fully understood, but there are several hypotheses. The dermatophytes could secrete a specific protease or keratinases which decompose the substrate. Kunert (1972b) suggested that dermatophytes denature keratin by enzymatic reduction of cystine bonds which form the basis of keratin resistance. The dermatophytes produce sulphite by oxidizing cystine even during growth of hair which splits disulphide bonds of keratin. This denatures the keratin and makes it digestible to exoproteases of dermatophytes.

Dermatophytes attack the keratinous tissue as a parasitic form, while it has been shown that *T. mentagrophytes* is capable of causing extensive breakdown of keratin in the saprophytic stages. Baxter and Mann (1969)

examined the pattern of invasion of human hair *in vitro* by three dermatophytes (*T. mentagrophytes, T. rubrum*, and *T. ajelloi)* and found variations in the keratinolytic ability of these species. *T. mentagrophytes* was the most keratinolytic and was seen to breakdown the hair keratin. Large numbers of granules and mitochondria were observed in *T. mentagrophytes* which were not observed in the other species and the authors suggested that this could be due to the higher enzymatic activity of *T. mentagrophytes.* Mercer and Verma (1963) looked at the invasion of sterile clipped human hair by *T. mentagrophytes in vitro.* They also found that the process of hair invasion involved an enzymatic breakdown of keratin bundles, with complete absence of the keratin bundles as seen in the present study. *T. mentagrophytes* was seen to digest the endocuticle, thus detaching the cells from the underlying cortex. Similar findings were observed in the present study where evidence of damage to the endocuticular layer was seen and the cuticular cells had detached from the underlying cortex. In both of the above studies the dermatophyte was initially seen growing intercellularly followed by intracellular growth . In the present study there was no evidence of intracellular growth as most of the fungal elements were seen growing intercellularly.

In this study germlings of *T. mentagrophytes* were seen to penetrate under the cuticle and in between the layers of cuticular cells. Daniels (1953) observed on the surface of hair, frond-like mycelium which appeared to be responsible for cortical erosion and suggested that it might also be the agent of cuticle lifting. It was suggested that *M. canis* was capable of digesting human hair keratin enzymatically and keratinases have been isolated from different dermatophytes. It has been shown that keratinases of *T. mentagrophytes* are capable of digesting hair (Yu *et al.,* 1969). Barlow and Chattaway (1955) described the stages of hair invasion by dermatophytes as (i) cuticle lifting, (ii) cortical erosion, (iii) perforation by penetrating organs. To this English (1963) added (iv) colonization of the medulla. Three of these stages were recognised in this study, but they overlapped one another during the process of hair

infection with *T. mentagrophytes*. Penetrating organs were not seen as they are a feature of saprophytic growth of *T. mentagrophytes* on hair.

Keratinophilic dermatophytes grow in a filamentous form during *in vivo* infection on hair, however, when growing *in vitro* on keratinized tissue, such as hair they produce flattened fronds of hyphae or eroding mycelium on the surface layers of these tissues, and a perforating organ develops from these fronds to penetrate the keratinized tissue (English, 1963; Raubitschek and Evron, 1963). The process of the degradation of keratin in hair and other natural substrates seems to be a result of both mechanical action of the dermatophytes (Baxter and Mann, 1969) and the enzymatic proteolytic activity of keratinases secreted by the fungi (Yu *et al*, 1972).

Kunert and Krajci (1981) studied the process of hair invasion by *M. gypseum in vitro* and found that the process of keratin degradation had features of enzymatic breakdown, with some evidence of mechanical effect of the hyphae on the cuticular cells. Shelley *et al.* (1987) suggested that the cuticle acts as a barrier against fungal invasion and is not vulnerable to the keratolytic enzymes. In the present study germlings were seen penetrating under and in between the cuticular cells and it is possible that this may be due to a mechanical action of the germlings though there was evidence of enzymatic damage to the cuticular cells in the present study. It appears that the process of hair invasion is due to a combination of both mechanical and chemical forces, one augmenting the other.

In the present study growth of the hyphae was directional, downward towards the hair bulb and fungal elements were seen in the hair cortex. Kligman (1955) reported the downward growth of intrapilar hyphae in tinea capita due to *M. canis* and they terminated abruptly at the upper limit of the keratogenous zone. Okuda *et al.* (1989 and 1991) have demonstrated fungal element of *M. canis* and *T. rubrum* in the hair cortex from lesions of tinea capitis.

6:6:3 Inhibitory effect of terbinafine on hair invasion by *T. mentagrophytes.*

Hair follicles in the presence of terbinafine which was incorporated in the medium it was observed that germination was seen to commence on the surface of the follicle but inhibition of further hyphal extension was evident. The results strongly suggest that sub-inhibitory concentrations of terbinafine profoundly affect the normal growth of the arthroconidia and hyphae of *T. mentagrophytes.*

Terbinafine is an effective short-term therapy for tinea capitis (Haroon *et al.*, 1992). Following oral administration terbinafine achieves a concentration of 2.6 ug/g of tissue in the hair, while in the sebum it achieves high concentrations up to 45.1μg/ml and continues to concentrate in the sebum after discontinuation of the drug (Faergemann *et al.*, 1990). These levels are far above the MIC's of terbinafine against dermatophytes. It is possible that this high level of the drug in and around the hair follicle and the fungicidal action of terbinafine may be responsible for the rapid response of tinea capitis to this drug.

In the present study it was observed that terbinafine in low concentrations acted as a barrier against further invasion of the hair by dermatophyte germlings. Systematically administered antifungal agents are most probably incorporated into the hair shaft and hair root sheath as well as excreted by sebaceous glands and sweat glands, forming a chemical barrier for the fungi. Gentles *et al.* (1959) showed that griseofulvin was incorporated in the keratinous tissue which contains a band of resistant keratin thus making the hair resistant against fungal degradation. After a few doses of griseofulvin the lower portion of hair shaft was free from dermatophytes and the hyphal tips of fungi were malformed. Further studies with griseofulvin have shown it can be extracted from hair by means of methanol and from the skin by means of water. This makes it probable that griseofulvin is not chemically bound, but located in the intercellular substance (Gentles and Barnes, 1960). Similar studies have

not been conducted with terbinafine but it is probable that this drug is deposited in keratinous tissues. Finlay (1992) showed that terbinafine rapidly penetrated the nail plate and persisted in the nail following cessation of oral therapy. Terbinafine may be inhibiting the secretion of keratinolytic enzymes secreted by *T. mentagrophytes*. It has been shown that dermatophyte keratinolytic enzymes activity plays a important role in hair infections and antifungal drugs which are capable of inhibiting ergosterol synthesis may also inhibit fungal keratinolytic activity (Abbink *et al.*, 1985).

In the present study adherence of arthroconidia to the non-keratinized part of the hair, including the hair bulbs was observed. Adherence of arthroconidia was also seen to terbinafine-exposed hair follicles. Arthroconidia are known to adhere to keratinized tissues, though pre-exposure of these tissues to terbinafine does not prevent adherence as it is possible that the amount of drug released from the corneocytes in these tissues is not enough to inhibit this process. Arthroconidia which are damaged by terbinafine failed to germinate (Rashid *et al.*, 1993).

Morphological modification seen in arthroconidia and hyphae of *T. mentagrophytes* growing on terbinafine-exposed hair showed drug-induced pores in arthroconidia, dilated and swollen hyphae. Similar morphological changes have been described with terbinafine on arthroconidia and germ tubes growing on the stratum corneum (Rashid *et al.*, 1993). They showed, at low concentrations, dilated germ tubes, while at higher drug concentrations, the germ tubes were collapsed and showed exfoliation of the surface layers with cell lysis. Arthroconidia showed small pores and extensive craters. When *T. mentagrophytes* was cultured on solid media containing terbinafine, swelling and ballooning of the hyphae and bulbiform blunt hyphal ends were seen (Nishiyama *et al.*, 1991). It is more likely to observe changes like dilated or swollen hyphae and club shaped ends at lower concentrations of the drug due to the growth inhibition effect, while at higher concentrations there is a fungicidal effect.

The new test model system described in this study can serve as a useful model for the detailed study of the effects of antifungal drugs on the invasion of hair by dermatophyte fungi. It can be used as an alternative to animal models for the pre-clinical evaluation for new antifungal agents in dermatophytosis.

6:7 Conclusion

A unique attempt was made in this study, to follow the course of invasion into viable human hair follicle by the dermatophyte *T. mentagrophytes* and to describe the use of an organ culture system of hair follicles to study antifungal drugs. The success of the experimental invasion described here is thought to be due to the production of the parasitic phase of dermatophytes. The organism seem capable of attacking the hair only if they multiply in a form morphologically similar to that of their parasitic phase. The process of hair invasion in this study had similarities with the natural disease. The present study contains morphological evidence to implicate both mechanical pressure and keratin dissolution as invasive mechanisms of dermatophytes in hair. The results strongly suggest that sub-inhibitory concentrations of terbinafine profoundly effect the normal growth and induce degenerative changes in the arthroconidia and hyphae of *T. mentagrophytes*. This model system seems to be a promising experimental system for studying the process of hair invasion by dermatophytes. It seems to be useful for the *in vitro* assessment of the effects of antifungal drugs on the morphological transformation of arthroconidia on hair and can be used as an alternative to animal models for the pre-clinical evaluation of antifungal agents.

Chapter 7

Growth of dermatophyte fungi on a human living skin equivalent and its use for the evaluation of antifungal drugs

7:1 Summary

The living skin equivalent model was used to study the growth of dermatophyte fungi and to evaluate the model for assessment of the activity of antifungal agents. One strain of *T. mentagrophytes*, and a living skin equivalent prepared by seeding foreskin keratinocytes onto forearm fibroblast-contracted collagen lattices was used. Arthroconidia were inoculated on the skin equivalent with and without terbinafine present. In the absence of terbinafine growth of the fungus was seen on the stratum corneum and was subsequently seen penetrating down into the dermis. Where terbinafine (0.01–10 mg/L) had been incorporated in the model, inhibition of growth was apparent and terbinafine was seen to act as a barrier against fungal invasion of the dermis. This study demonstrated that the skin equivalent model is ideally suited for the growth of dermatophytes, and that certain serum factors may limit dermatophyte growth to the stratum corneum. This model is a promising *in vitro* experimental system for testing antifungal drugs and as an alternative to animal testing.

7:2 Introduction

Living skin models can be reconstructed *in vitro* by a number of methods. The various approaches have in common that keratinocytes are cultured at the air/liquid interface attached to a substrate which includes some mesenchymal element. A system has been devised in which epidermal keratinocytes from human neonatal foreskin can be serially cultivated, forming colonies of stratified squamous epithelium (Rheinwald and Green, 1975). The cultured cells retain differentiation properties, forming a multilayered keratinizing epidermis with desmosomes, tonofilaments and hemidesmosomes (Bell *et al.*, 1983). The human skin equivalent consists of two components: the dermal equivalent which is prepared first, consisting of fibroblasts in a collagen

matrix that is contracted and modified by the resident cells and an epidermal equivalent which is recreated by applying a suspension of keratinocytes on to the dermal equivalent, becoming stratified at the air/liquid interface. Histologically, the two layered model bears a very close resemblance to a section through human tissue (Bell *et al.*, 1983). Sun and Green (1978) have shown that human epidermal cells grown in culture synthesise abundant keratins which have many similarities with those of the stratum corneum. About 20% of all the protein of living cultured keratinocytes consists of keratins as compared with over 85% of the stratum corneum. *In vitro* cultured keratinocytes have been used as epidermal grafts to cover deep and extensive burn wounds and chronic ulcers (Parenteau *et al.*, 1991). Cultured epidermis is used for a number of applications, including its use as a model for toxicity testing, basic research, percutaneous absorption, and UV radiation effects.

In the skin equivalent the cells tend to form a well differentiated epidermis, with a stratum corneum and granulosum including keratohyalin and membrane–coating granules and because the upper layers are exposed, materials can be applied directly to the "skin's surface". Monolayered cells have been used to study *in vivo* or *in vitro* the application of pharmacological agents, but with the development of a skin equivalent model, a system is provided for the study of cellular responses at the tissue and organ levels *in vitro* (Coulomb *et al.*, 1984).

Dermatophytes are a group of keratinophilic fungi with the ability to invade and parasitize the non living cornified layer. In the skin they are localised to the stratum corneum. The failure of dermatophytes to invade the deeper living, non keratinous layers of the skin is not because they need keratin, but that they cannot survive in the presence of serum (King *et al.*, 1975). Only in the keratinized tissue are dermatophytes protected from serum inhibitory factors. Previous experiments with dermatophytes and cultured skin are few. Blank *et al.* (1959) studied the growth of dermatophytes in pellet form on cultured human skin and demonstrated the localisation of dermatophytes to

the outer keratinized structures only. This work suggested that it was as a result of a fungistatic effect of serum. There are few satisfactory *in vitro* experimental model systems in which to study the colonization of skin by dermatophyte fungi, including the germination and hyphal extension of such fungi, and their response to various antifungal agents. As the living skin equivalent has a very close resemblance to the normal tissue and as there is no human serum present, it was used in this study. Because of its use in pharmacological studies antifungal drugs can be incorporated in this model to assess their activity.

7:3 Aim of the study

This study was undertaken to examine the establishment and growth of *T. mentagrophytes* arthroconidia on a human living skin equivalent and to assess the efficacy of this model for investigating the activity of antifungal drugs.

7:4 Materials and methods

7:4:1 Organism and stock cultures

One strain of *T.mentagrophytes* was used in this study. This strain was the same as described in Chapter 4, section 4:4:1. Subcultures were maintained on GPA and incubated at 28°C for 10 days to obtain an optimal yield of microconidia.

7:4:2 Production and preparation of arthroconidia

Arthroconidia were produced and prepared to a concentration of 5×10^4 arthroconidia per ml as described in Chapter 2, sections 2:4:3 and 2:4:4.

7:4:3 Preparation of living skin equivalent

Human foreskin was cut into strips, the subcutaneous fat was removed and the strips were placed with the dermal side down in 0.5% (w/v) dispase in phosphate buffered saline (PBS) and incubated at 4°C overnight. The epidermis was then separated from the dermis, chopped up and placed in a sterile universal container. Then keratinocytes were dissociated by incubation for 3 min. in 0.05%(w/v) trypsin, 0.02%(w/v) EDTA in Ca^{2+} and Mg^{2+} – free PBS. The trypsin was then inactivated by the addition of Dulbecco's minimal essential medium supplemented with 10%(v/v) foetal calf serum (FCS). The suspension was then centrifuged for 3 min at 400g. Cells were resuspended in Dulbecco's minimal essential medium containing 10% FCS, prior to seeding on contracted collagen lattices.

7:4:3:1 Fibroblast culture

Human adult forearm fibroblasts were cultured in Eagle's minimal essential medium (MEM), supplemented with 10% FCS, L–glutamine (2 mM) and penicillin (100 units/ml) and streptomycin (100 μg/ml). The medium was then removed and the cell layer washed with Ca^{2+} and Mg^{2+} – free PBS/EDTA and the cells were detached by a brief exposure to0.05% trypsin / 0.02% EDTA in Ca 2+ Mg2+ – free PBS and the, trypsin inactivated by addition of serum – containing MEM. The cells were routinely split 1 in 5 into new flasks with fresh medium.

7:4:3:2 Collagen gels

Seven volumes of rat tail tendon collagen solution (3 mg/ml of 1/1000 acetic acid) were mixed with 2 volumes of 10 x MEM: 0.34 M NaOH (2:1 v/v) and the pH finely adjusted to a pH of 7.2 with 0.34 M NaOH.

One volume of FCS – containing fibroblasts (7.5 x 10^5 cells/ 10 ml of collagen gel solution) was then added, mixed thoroughly, and 3.0 ml aliquots were added to 35 mm sterile petri dishes. The gels were allowed to set for 15 min, at 37°C, the complete medium added and the gels mechanically detached from the dishes and incubated at 37°C in a 5% CO_2 in air humidified atmosphere. Following 6 days incubation the fibroblasts had contracted and reorganised the collagen to form a compact tissue – like dermal equivalent.

The keratinocyte cell suspensions were then seeded onto the contracted collagen lattices in submerged cultures for 4 days, after which the models were raised to the air/liquid interface, where a highly differentiated stratified epidermis formed following a further 6 days incubation.

7:4:4 Preparation of terbinafine solutions

Solutions of terbinafine were prepared as described in Chapter 3, section 3:4:4. A 100 mg/L stock solution was prepared and by serial dilution a concentration range (0.001 – 10 mg/L) was prepared.

7:4:5 Inoculation of living skin equivalent

The living skin equivalent model medium was washed twice in CO_2 – independent tissue culture medium, containing FCS, L–glutamine and penicillin and streptomycin. A 5 µl inoculum of a 5 x 10^4 arthroconidia per ml suspension was inoculated onto the skin equivalent and then incubated for varying lengths of time at 28°C. The medium was changed after every 48h incubation. This procedure was carried out in a UV light–sterilised hood under aseptic conditions.

7:4:6 Assessment of living skin equivalent invasion by *T. mentagrophytes* and antifungal activity of terbinafine.

Gross examination was carried out to assess fungal growth. For light microscopy, the living skin equivalent was set up as in section 7:4:5 and then fixed in 4%(v/v) formaldehyde for H and E and PAS staining after incubation for the appropriate time intervals.

For scanning electron microscopy skin equivalents were set up as in section 7:4:5 and after growth had proceeded for the desired time they were fixed and processed for scanning electron microsccpy as in Chapter 2, section 2:4:9 .

For assessing the antifungal activity of terbinafine, low concentrations (0.01–10 mg/L) of terbinafine were incorporated in the CO_2–independent tissue culture media and then incubated at 28°C for up to 7 days. Gross examination was done and H and E and PAS sections were examined by light microscopy. Scanning electron microscopy was carried out to observe morphological changes in the dermatophyte.

7:5 Results

7:5:1 Gross examination

On gross examination control skin equivalent models gave a whitish growth on their surface around 4 days of incubation. Where terbinafine had been incorporated in the medium or applied to the surface of the model no growth was seen and a clear surface was visible.

7:5:2 Light microscopy

Control H and E sections showed a well stratified epidermis including a well developed stratum corneum (Fig 7:1). Sections fixed around 72h showed evidence of germination of arthroconidia with the fungal elements growing on the stratum corneum, with evidence of horizontal and perpendicular penetration on the stratum corneum by the germ tubes (Fig 7:2). There was no penetration below the stratum corneum at this stage. Sections fixed around 6 days showed extensive growth of fungal elements on the stratum corneum and penetration of the epidermis, replacing most of it with mycelium. Fungal hyphae were observed growing throughout the epidermis and lying on the upper dermis as a result of penetration from the epidermis (Fig 7:3). The epidermis at the extent of mycelial growth appeared relatively normal.

Where terbinafine had been incorporated in the medium, growth inhibition was seen in the range of 0.01– 1mg/L. At a concentration of 0.01 mg/L a barrier effect of the drug was observed, with mycelial growth taking place in the epidermis, with only a few hyphae lying in the upper dermis as compared to controls where a large number of hyphae were scattered in the dermis. At a drug concentration of 0.1 mg/L no fungal elements were observed in the dermis; they were localised to the epidermis(Fig 7:4). At 1mg/L there was very little growth seen in the epidermis, the mycelium appeared compacted with no growth evident in the dermis. At higher drug concentrations no growth was seen on the models.

It was observed that skin equivalents which had a well formed multilayered epidermis with a stratum corneum exhibited more extensive growth of fungal elements on their surface. Arthroconidia formation was observed in the mycelium growing on the epidermis.

7:5:3 Scanning electron microscopy

On SEM it was observed that arthroconidia were adhering to the surface of the skin equivalent. Adherence of ungerminated arthroconidia to the keratinocytes and the collagen fibrils was observed. Adherence to keratinocytes was particularly prominent with a depression formed where arthroconidia binding had occurred. Some arthroconidia were seen lying deep in the model entangled in the collagen fibrils. A mixed picture of arthroconidia of various shapes and sizes was observed at 48h with the arthroconidia varying in the range of 8–10 μm in size. Little germination of arthroconidia was seen at this stage (Fig 7:5). By 6 days, germination of arthroconidia had taken place with hyphal extension occurring on the surface and side branches appearing with a well formed mycelium. Penetration of the model was seen by the germlings which were observed growing in through pores and cavities and germlings crossing depressions in the model (Fig 7:6). The germlings were seen growing very close on the surface of the model. Germlings were seen to grow very well on collagen matrix and were seen tunnelling through them and penetrating pores in the collagen lattices. Segmentation and arthroconidia formation was seen in the hyphae (Fig 7:7).

Where terbinafine 0.1 mg/L had been incorporated in the medium, adherence and germination of arthroconidia was seen. Hyphae were seen growing on the model, but were dilated and appeared empty. No true mycelium was formed since the continuity was broken with areas of sparing seen on the surface of the model due to growth inhibitory effect of the drug. The hyphae exhibited increased segmentation and there was evidence of distortion of the hyphae (Fig 7:8).

Figure 7:1a

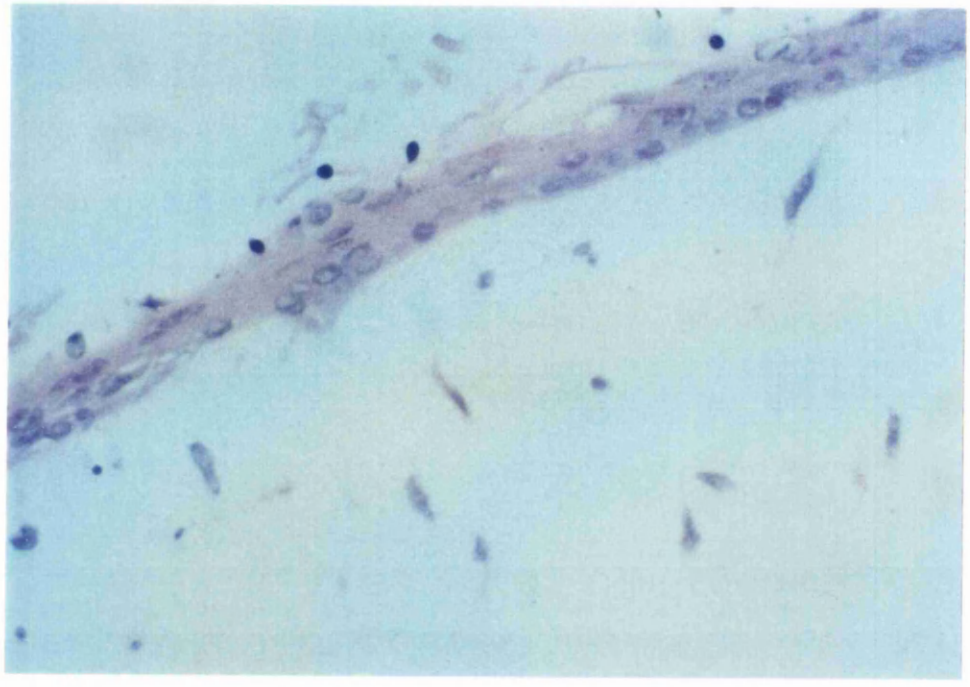


Figure 7:1b

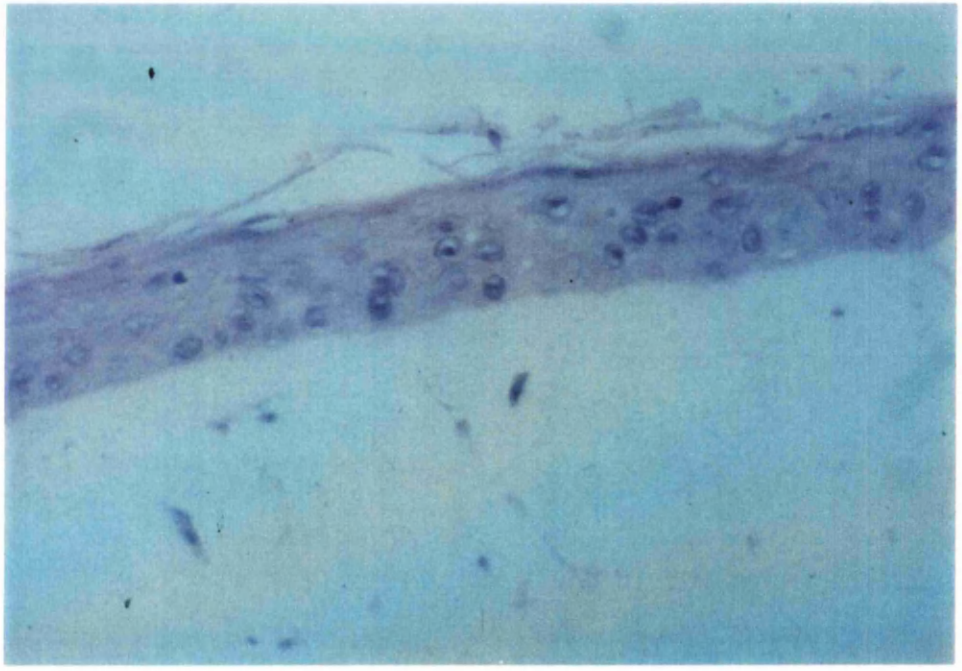


Figure 7:1 Development of the living skin equivalent following seeding of a single cell suspension of keratiocytes on to a fibroblast–contracted collagen lattice (a)–incubation as a submerged culture for 5 days (b) – incubation for a further 3 days at the air/liquid interface (c) – incubation for 6 days at the air/liquid interface. H and E stain X 250.

Figure 7:1c

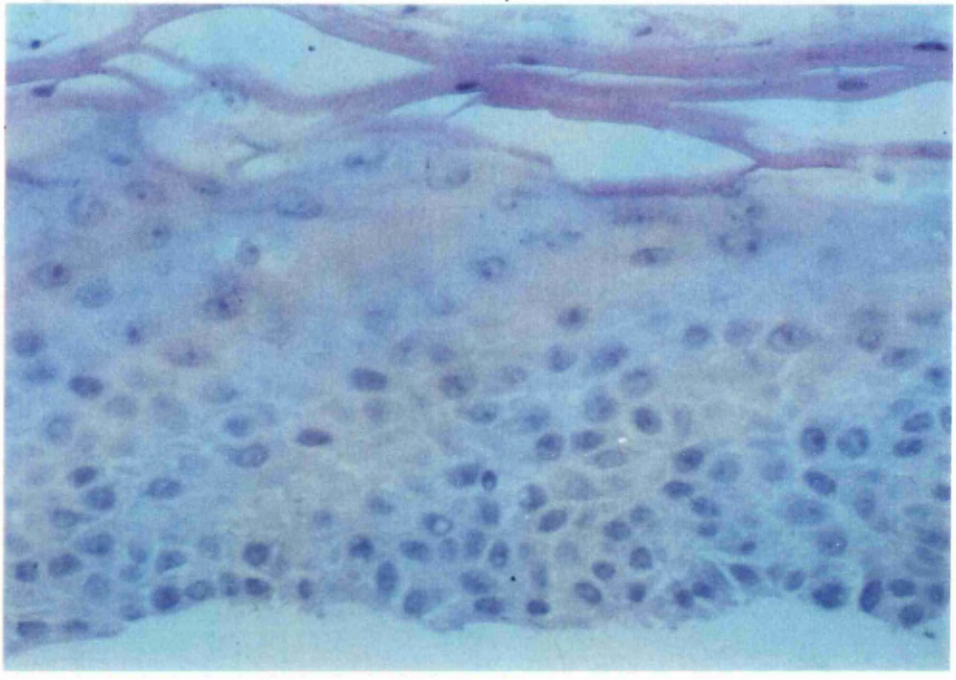


Figure 7:2a

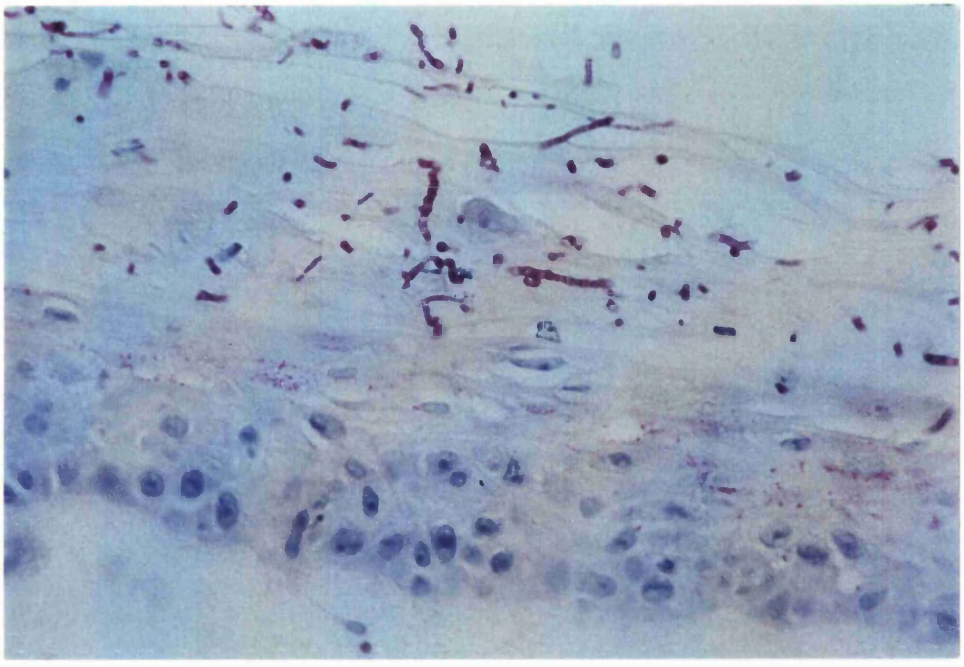


Figure 7:2b

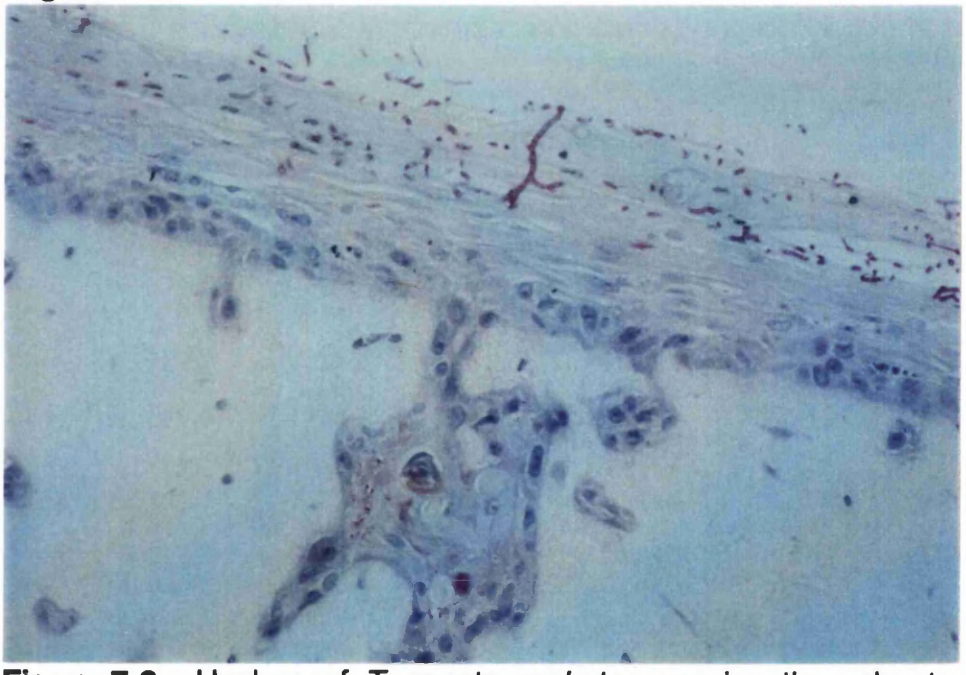


Figure 7:2 Hyphae of *T. mentagrophytes* growing throughout the layers of the stratum corneum after incubation for 72h at 28°C (a) Note the perpendicular and lateral penetration of the hyphae through the layers of the stratum corneum X 400 (b) X 250. PAS stain

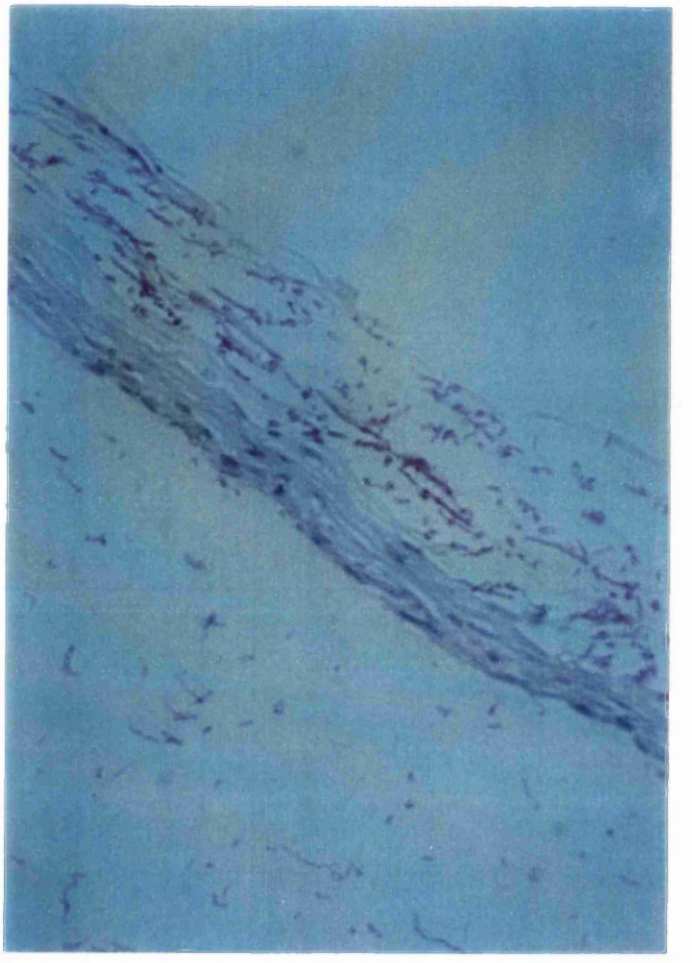


Figure 7:3 Fungal elements of *T. mentagrophytes* growing on the stratum corneum and penetrating into the dermis, following 6 days incubation at 28°C X 250. PAS stain.

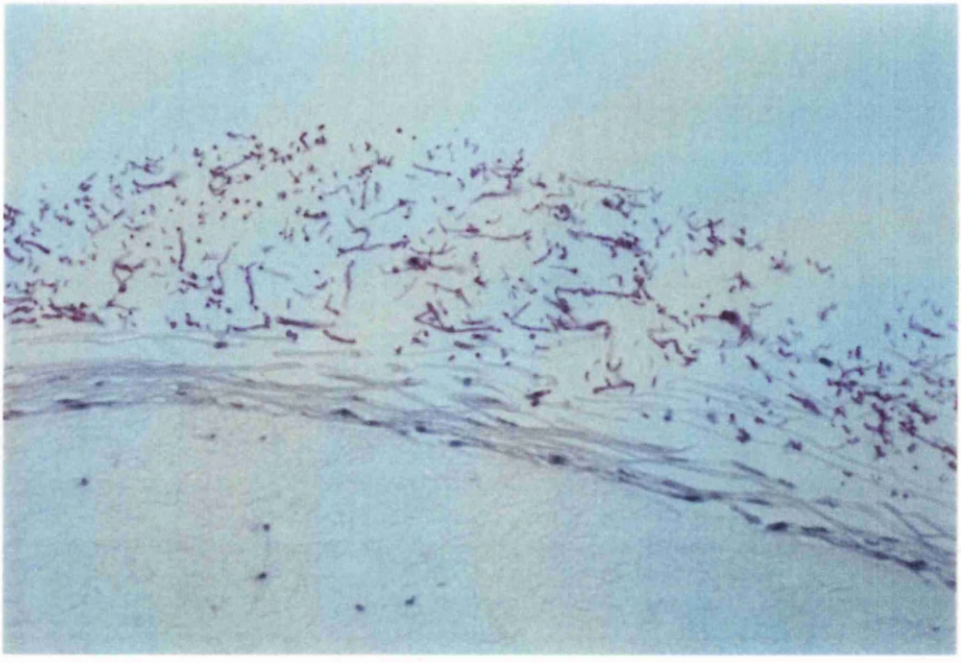


Figure 7:4 Fungal elements of *T. mentagrophytes* are growing on the epidermis but not penetrating into the dermis following 6 days incubation at 28°C and 0.1 mg/L terbinafine incorporated in the model X 250. PAS stain.

Figure 7:5a

Figure 7:5 Scanning electron micrograph of *T. mentagrophytes* arthroconidia lying on the surface of a skin equivalent after 48h incubation at 28°C. (a) Two arthroconidia seen adhering to the stratum corneum of the skin equivalent X 4400 (b) Four arthroconidia adhering to keratinocytes on the surface of the skin equivalent X 3000 (c) a depression is formed where the arthroconidium is lying on the keratinocyte. Note the smooth surface X 6000 (d) an arthroconidium (arrow) lying entangled in the collagen fibrils of the skin equivalent X 28000.

Figure 7:5b

Figure 7:5c

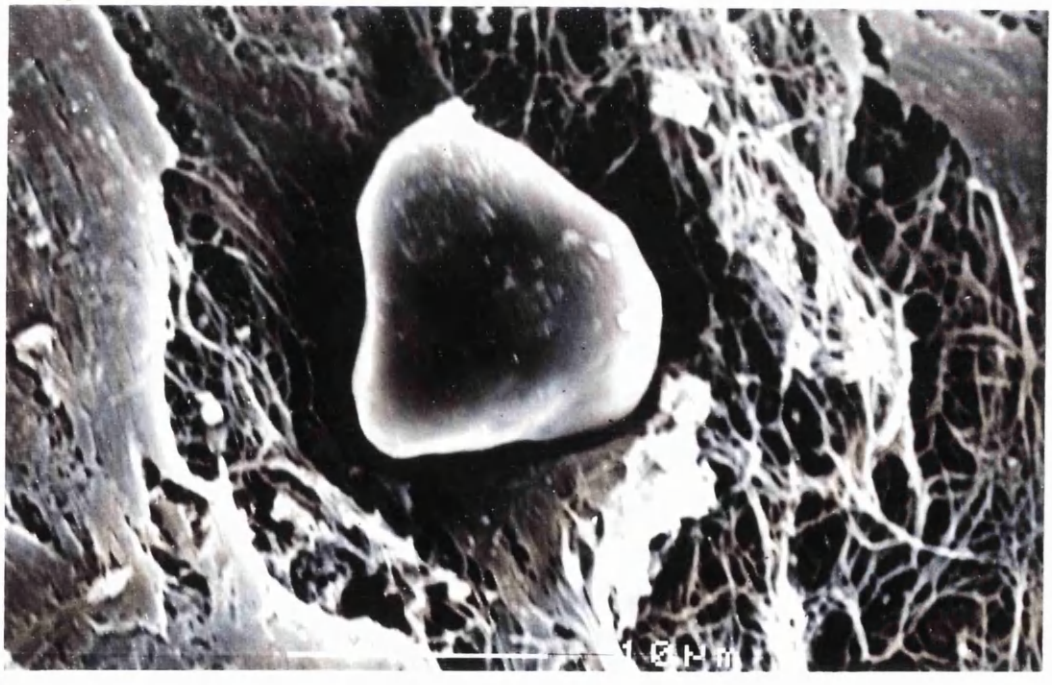


Figure 7:5d

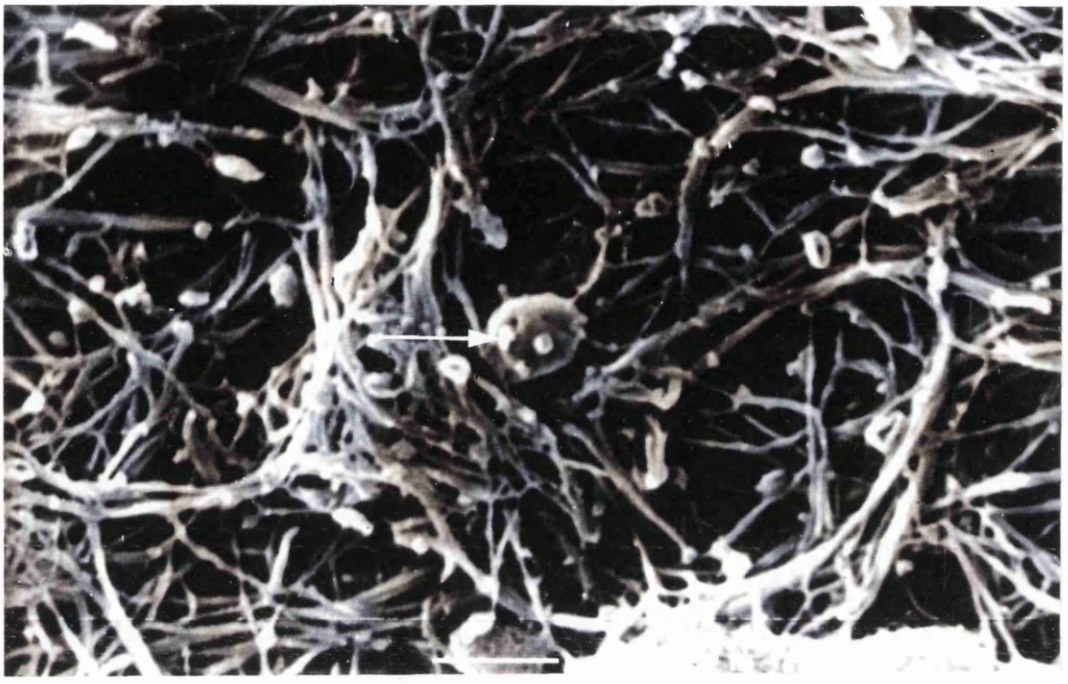


Figure 7:6a

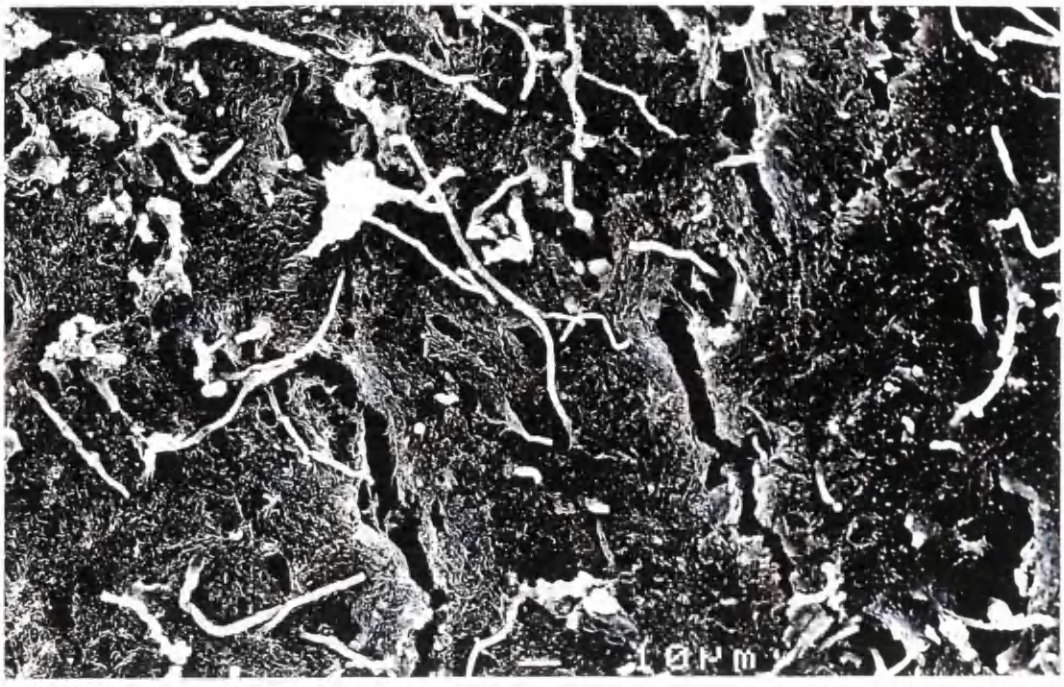


Figure 7:6b

Figure 7:6 (a) Scanning electron micrograph of *T.mentagrophytes* hyphae penetrating through crevices and pores on the surface of the skin equivalent X 900 and (b) bridging over crevices X 3600.

Figure 7:7a

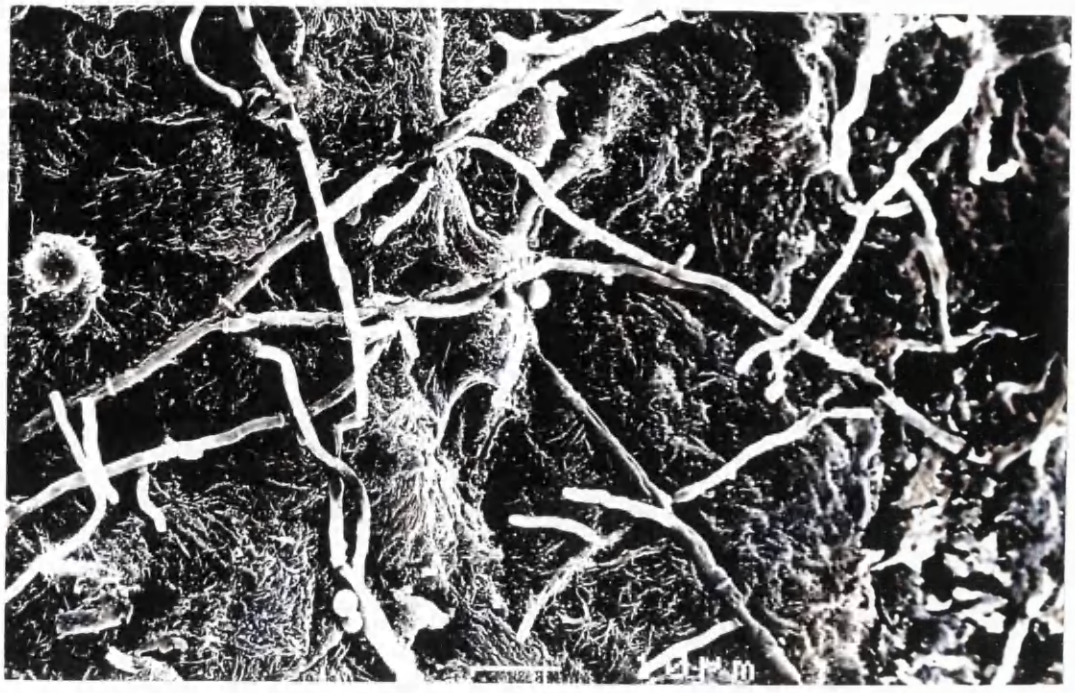


Figure 7:7b

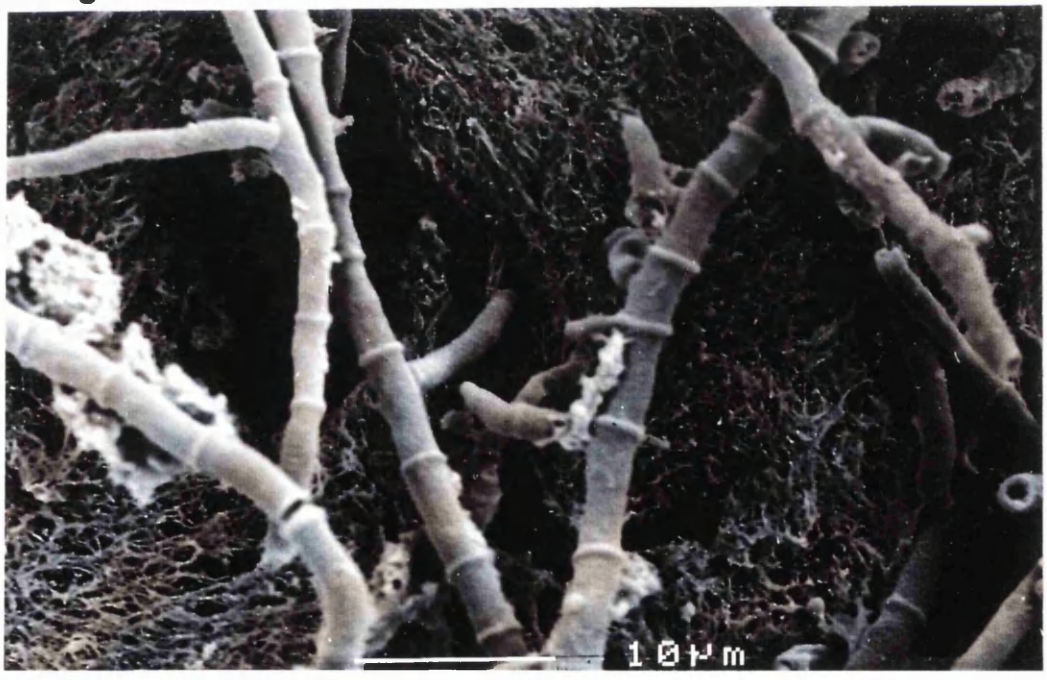


Figure 7:7 Scanning electron micrograph of penetration of contracted collagen lattices by germlings of *T. mentagrophytes* (a) Note the tunnelling phenomenon of the hyphae on the surface X 2000 (b) dermatophyte hyphae are seen penetrating contracted collagen lattice through pores on the surface of the lattices. Note the segementation of the hyphae. X 4600.

Figure 7:8a

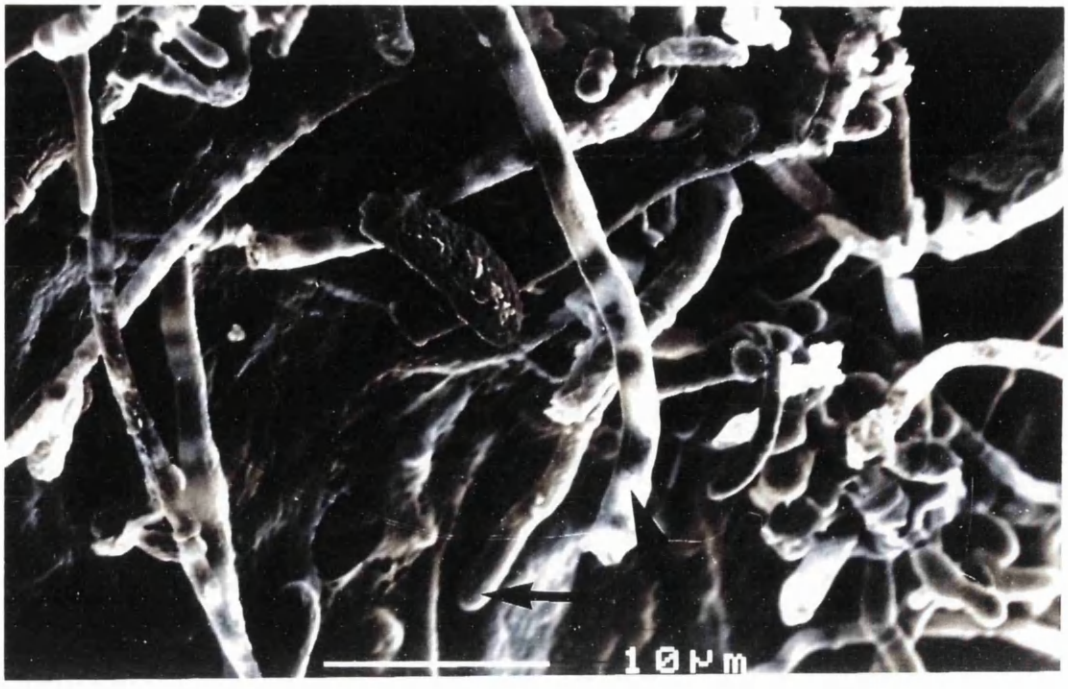


Figure 7:8b

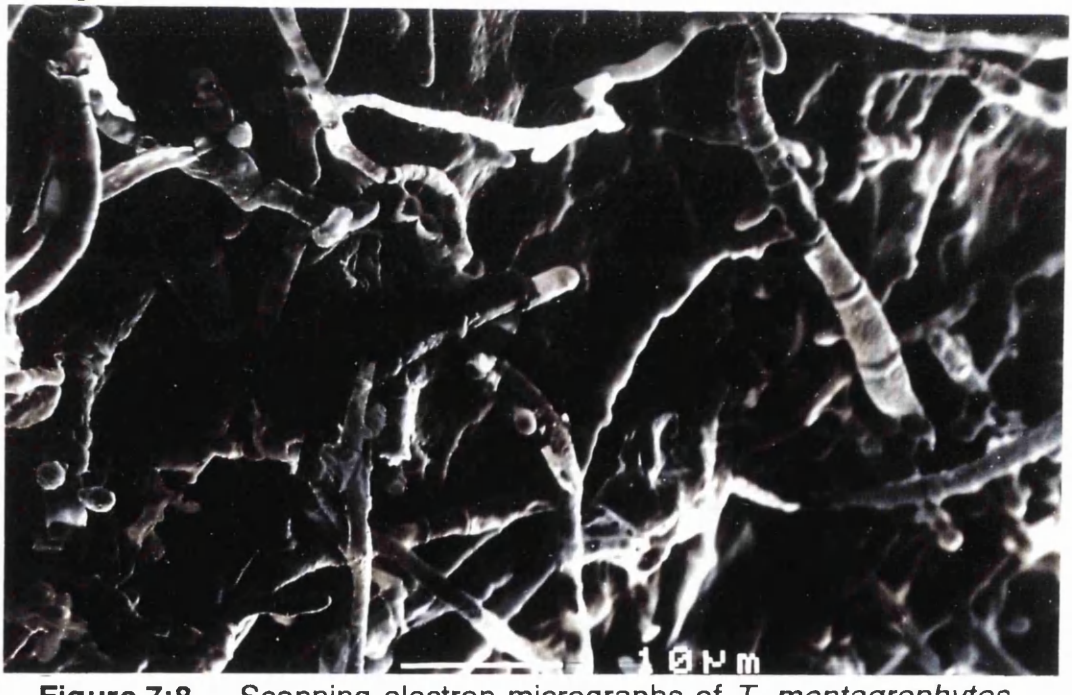


Figure 7:8 Scanning electron micrographs of *T. mentagrophytes* hyphae following exposure to 0.1 mg/L terbinafine in the skin equivalent. (a) Note the dilated and empty looking hyphae with swollen tips (arrow) X 5000 (b) there is increased segementation of the hyphae which appear unhealthy X 3600.

7:6 Discussion

7:6:1 Growth of *T. mentagrophytes* on human living skin equivalent

It was possible to reconstruct a living skin equivalent *in vitro* by seeding keratinocytes on a collagen gel which had human fibroblasts incorporated in it. This model was used to study the growth of the dermatophyte *T. mentagrophytes*. The results of this study show that adherence and germination of arthroconidia takes place on the surface of the model and the germlings grow in a manner which has a close resemblance to their growth *in vivo* on the stratum corneum (Richardson and Aljabre, 1993). Adherence of arthroconidia to corneocytes has been shown to be an early step in the establishment of dermatophyte infection. The arthroconidia secrete some form of adhesin which helps in attaching them to keratinocytes, though the exact nature of this material is not known. It has been observed that the contact between dermatophyte arthroconidia and corneoctyes is very close with a loose extracellular material between the two (Aljabre *et al.*, 1993; Rashid *et al.*, 1993). In the present study a very close and firm adherence of arthroconidia was seen to the surface of the model to the keratinocytes and the collagen matrix suggesting that the arthrocondia may be secreting some adhesive material.

Germination of arthroconidia and growth of germlings was seen on the surface of the model, with germ tubes penetrating the surface through crevices and pores. Aljabre *et al.* (1992a) described germination of arthroconidia and hyphal penetration of the stratum corneum as crucial factors in the establishment of infection, and suggested that germ tubes, by penetrating the stratum corneum, and lying in between or attached to corneocytes, are thus capable of holding the mycelium on the stratum corneum. It was observed that the germlings were penetrating through pores in the model and bridging over crevices on the surface of the model, described as thigmotropism or contact sensing. This phenomenon has not been described before for dermatophytes.

Sherwood *et al.* (1992) described this phenomena with the germ tubes and hyphae of *C albicans* grown on nucleopore filters and suggested that contact sensing in *C. albicans* may be a phenomena which occurs in response to their invasion of mucosal surfaces. Dermatophytes are known not to invade mucosal surfaces and the most important feature offering protection to the skin and deeper tissues from invasion is an intact stratum corneum. This phenomena may be occurring when there are cracks and crevices in the stratum corneum (Richardson, 1991) which offer the dermatophyte hyphae a route to grow down and in between the cells feeding on the intercellular material as it penetrates deeper. Alternatively, breaks in the stratum corneum expose the hyphae to the living layers of the skin and certain factors present in these living layers attract the hyphae in their direction.

In the present study it was observed that with longer incubation, hyphae were seen penetrating down from the stratum corneum to the epidermis and then into the dermis. It is thought that dermatophytes invade and proliferate almost exclusively in the keratinized portion of the skin, hair and nails, where it is assumed that they possess keratinolytic enzymes and digest keratin. Only rarely do they penetrate deeper tissues. Blank *et al.* (1959) studied the growth of dermatophytes in pellet form on cultured human skin which was placed on agar and they showed the localisation of dermatophytes to the outer keratinized layers in the presence of serum and when no serum was present, invasion by mycelial elements of the epidermis and dermis was seen. The penetration of germ tubes to deeper layers of the stratum corneum has been described in experimental dermatophytosis in guinea pigs (Fujita and Matsuyama, 1987). King *et al.* (1975) suggested that the reason for dermatophytes being confined to the keratinized tissues was because they were protected from an inhibitory factor present in serum. They identified this factor, serum inhibitory factor (SIF) as unsaturated transferrin. Lorincz *et al.* (1958) showed that dermatophytes were inhibited when implanted in the abdomen of healthy mice but grew vigorously when transferred to culture medium. It has been demonstrated that

dermatophytes grow profusely in all layers of viable full-thickness skin explants maintained in short term tissue culture, and growth of the dermatophytes is prevented by bathing the explants in fresh human serum.

In the present study no human serum was used and only a small amount of foetal calf serum was added to the medium. It appears that the penetration of the deeper layers took place due to the absence of an inhibitory factor which is present in human serum.

It was observed that the hyphae of *T. mentagrophytes* were segmenting and arthroconidia were forming, suggesting that the dermatophyte was capable of completing its full life cycle on the skin equivalent model.

7:6:2 Human living skin equivalent, a novel *in vitro* model for evaluating antifungal drugs

In this study the skin equivalent model was tested as a system for evaluating antifungal drugs. The results showed that low drug concentrations of terbinafine acted as a barrier against fungal elements penetrating the dermis while at higher concentrations there was a fungicidal effect of the drug.

Monolayered cells have been used to compare cellular responses *in vitro* with cells *in vivo*, but the cellular responses observed in de-differentiated cells in monolayer culture are not at all predictive for the response of the same cells *in vivo*. These differences seem to be due to cell-cell and cell-matrix interactions existing *in vivo* which are responsible for cell differentiation. The reconstructed epidermis has been successfully used to study the effects of retionic acid on regulation and differentiation of the epidermis (Regnier and Darmon, 1989).

It was observed that terbinafine was acting as a barrier to the invasion of the dermis by fungal elements. It has been shown that terbinafine rapidly accumulates in the stratum corneum mainly by diffusion from the vascular system through the dermis - epidermis (Lever *et al.*, 1990). High

concentrations of this drug are found in the sebum produced by sebaceous glands and in and around the hair follicle which are both dermal structures (Faergemann *et al.*, 1990). Terbinafine is lipophilic and it is possible that this drug is somehow deposited in the dermis hence acting as a barrier to further proliferation of the fungus. It is known that terbinafine strongly adheres to keratin and prevents further invasion of nail and hair by *T. mentagrophytes* as seen by the previous work in Chapters 5 and 6.

7:7 Conclusion

This is the first study using the living skin equivalent to study the effects of antifungal drugs on dermatophyte fungi. The results show that there are very close similarities between growth of dermatophytes on a living skin equivalent and the stratum corneum with the exception of invasion of the dermis which is not seen in natural disease. It seems that the living skin equivalent has all the nutrients and signals required for the growth of dermatophytes. Based on these findings it seems to be a suitable model for the study of the pathogenesis of dermatophytosis. The results of this study are in keeping with those of previous workers that a factor in human serum is responsible for localising the growth of dermatophytes to the stratum corneum. The results support the contention that terbinafine is deposited in tissues, in particular the dermis and then acts as a barrier to further invasion of these tissues by dermatophytes. Although it is unlikely that one methodology will meet all the criteria for replacing animal testing, the results of this study shows that the living skin equivalent model appears to be a unique system for studying the biology of dermatophyte infections and represents when experimental conditions are carefully monitored, a predictive system for testing antifungal drugs. With multiple end points and striking structural similarities to human skin, the three-dimensional skin model makes a significant contribution to the quest for a useful *in vitro* test method for testing antifungal drugs.

Chapter 8

General Discussion

The morphological transformation of dermatophyte arthroconidia on keratinized tissues with and without terbinafine was studied. Adherence and germination of arthroconidia was followed by growth of the germlings on all of the normal tissues examined, that is stratum corneum, nail and hair. It was observed that there was early invasion of these tissues, which was seen by 24h. There was a close relationship between the keratinized tissues and the arthroconidia and germ tubes growing on it. On the stratum corneum, close adherence between the arthroconidial wall and corneocyte surface was seen with a fibrillar-floccular material in the intercellular space. The exact nature and origin of this material and its significance to adherence needs to be explored. Factors present in or released by corneocytes appear to be crucial in the establishment of dermatophyte infection on the stratum corneum. These factors require to be identified. There were similarities between the growth of dermatophyte fungi in these tissues and natural disease. There was evidence to suggest that the process of invasion of keratinized tissues is a combination of both mechanical and chemical forces, one augmenting the other. The exact nature of these forces and whether the damage to these keratinized tissues by the invading fungus is due to mechanical or chemical action or a combination of both is an area which needs to be looked into much further. The conditions described in this study simulated the physiological environment of dermatophyte fungi and hydration, maceration and occlusion do not appear to be essential for the establishment of infection. It seems that the dermatophyte can overcome the local factors present and invade these tissues through firm adherence and germination of arthroconidia followed by growth of germlings on and in between corneocytes thus establishing infection. The exact signals involved in the adherence, germination and hyphal extensions in these tissues needs to be investigated. Similarly, factors present in or released by the cells in these tissues and their role in establishment of infection has to be further examined.

It was observed that dormant arthroconidia were more susceptible to damage by terbinafine. More morphological changes were observed in the arthroconidia tested at lower drug concentrations than in the germ tubes. Furthermore, terbinafine partially inhibited germination of arthroconidia which suggested that once arthroconidia have become morphologically committed germination cannot be arrested. However, the finding that germ tube elongation was inhibited indicates that terbinafine has a delayed effect on cellular differentiation, which may have direct effect on the morphological transformation and invasiveness of dermatophyte fungi. Ultrastructurally, arthroconidia exhibited varying degrees of damage following exposure to terbinafine. Damage included a rough exfoliative surface to pores and craters. Similarly, germ tubes were damaged with pores, rough exfoliative surface and collapsed hyphae. These findings suggested that apart from inhibition of squalene epoxidase leading to squalene accumulation, terbinafine has a direct damaging effect on the cell wall and that the outer and inner layers of the arthroconidial cell wall are the initial targets of terbinafine action followed by alteration to the cytosol and intracellular organelles.

In the tissues, at the time of parasitism the dermatophyte mainly exists as arthroconidia. This study has shown that arthroconidia are extremely sensitive to terbinafine and this could explain the marked efficacy of terbinafine in dermatophytosis. The immediate clinical implications of this study are that prophylaxis of ringworm in endemic areas with terbinafine may also be highly effective since arthroconidia coming into contact with the keratinized tissues will be damaged and morphological transition to the hyphal form will be inhibited. Furthermore, by incorporation of the drug into the keratinized tissues, terbinafine can act as a barrier to further proliferation of the fungus.

The assessment of antifungal agents is fraught with several difficulties and to overcome them new experimental models for testing antifungal drugs have been described in this study. The advantage of these methods is that they simulate the physiological environment of the fungi. They are simple,

rapid, efficacious and inexpensive. The wider use of these methods for pre-clinical evaluation and screening of antifungal compounds, and their use in clinical practise to test for the efficacy of topical or a systemically administered antifungal drug are areas which have to be yet explored.

Appendix

I. Glucose peptone agar

Composition / 500ml distilled water

Glucose	20 gm
Peptone (Bacteriological, Difco)	5 gm
Agar (Bacteriological , Difco)	10 gm
All media were sterilized at 15lbs/in^2 for	20 min

II. Tween 80

Composition / litre distilled water

Polyoxyethylene (20) sorbitan mono-oleate	10 ml

III. Periodic acid – Schiff's staining

The D-SQUAMES discs were fixed in formaldehyde and then stained as follows

Periodic acid	20 min
Distilled water	5 min
Schiff's reagent	20 min

IV. Carbon dioxide independent medium (Gibco)

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