Model systems for the study of dermatophyte and non-dermatophyte invasion of human keratin

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Summary

Both dermatophyte and non-dermatophyte fungi can colonise and invade the keratin of skin, nail and hair. The fungal morphological forms that adhere to these keratinaceous substrates are most probably arthroconidia and hyphal fragments in association with exfoliated corneocytes. Towards a greater understanding of the ability of fungi to utilise keratin we have developed a series of experimetal models to stimulate the invasion of the stratum corneum, the human nail and the hair follicle. In many respects the mode of invasion mimicked human disease. The models were used to determine the bioavailability of terbinafine in keratinous substrata. Terbinafine prevented invasion of keratin by *Trichophyton mentagrophytes*. These models appear to be very suitable for the study of the mode of action of antifungal drugs used in the treatment of dermatomycoses.

Key words

Dermatophytes, Non-dermatophytic moulds, Dermatophytosis, Onychomycosis, *Trichophyton mentagrophytes*, Terbinafine

Stratum corneum is the outermost layer of skin. Its external aspect is in contact with the outside environment and its internal aspect is in contact with the sub-stratum corneum environment. Skin is sterile at birth but soon becomes colonised by a number of microorganisms which make up its flora. Dermatophytes are not considered part of this flora.

Dermatophyte fungi have been shown to have keratinolytic, other proteolytic and lipolytic activity [1]. Serine proteinases (urokinase and tissue type plasminogen activator) which are involved in extracellular protein catabolism have been found in dermatophytes and their release by dermatophytes was suggested to play a major role in the invasion of skin. Sulphitolysis, a process that denatures keratin non-enzymatically, has been found during dermatophyte- induced keratinolysis and suggested as a complementary mechanism to keratinolysis. Keratinase has been partially purified from and detected by immuno-electron microscopy in material from *tinea pedis* caused by *Trichophyton rubrum*. Using a fluorescent antibody technique keratinase has also been detected in biopsies from the skin of guinea pigs infected experimentally with *Trichophyton mentagrophytes*. Disintegration of hair thought to be caused by dermatophyte enzyme digestion has been shown in human scalp infection, experimental infection in guinea pigs and *in vitro*. Thus it seems that dermatophytes have a battery of enzymes able to digest different substrates in their habitat.

Dermatophyte fungi invade the stratum corneum. In the skin there are a number of conditions that favor the growth of dermatophytes while others do not. Conditions favorable for growth of dermatophytes include:

1. The stratum corneum is an avascular tissue composed of highly specialized but dead cells. It is distant from the bodies main defensive mechanisms.
2. The stratum corneum is well hydrated - water reaches it through eccrine sweating and transepidermal water loss. Skin temperature is cooler than body temperature; pH ranges from 5.5 to 6.7. Skin is exposed to the aerobic conditions of the atmosphere.
3. Stratum corneum is an agreeable tissue for growth of dermatophytes because it is composed of proteins, amino acids, lipids, carbohydrates and various trace elements, including iron.
4. Over some areas of the stratum corneum there are certain anatomical considerations which may enhance establishment of growth of dermatophytes. Firstly, hair on scalp may act as a trapping device for an airborne dermatophyte infection. Secondly, the hyponychial horny layer is covered by the distal portion of the nail plate and a groove is thus constructed which may also act as a trapping device for dermatophyte infective particles. Thirdly, the interdigital spaces of the toes, particularly the fourth, and the crural areas in males are naturally occluded and this may contribute to the fact that *tinea pedis* in most instances starts in the toes webs and *tinea cruris* is almost exclusively a male disease. Experimentally-induced occlusion will cause the hydrated stratum corneum to swell and develop multiple folds and allow accumulation of desquameted corneocytes on the surface of the stratum corneum. Therefore, it is likely that occlusion increases

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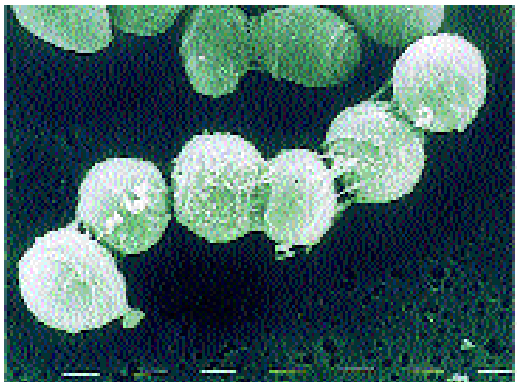

Figure 1. Scanning electronmicrograph of arthroconida of *Trichophyton mentagrophytes* grown for 10 days in 10% carbon dioxide at 37°C. The arthrospores appear mainly with a fibrilllar coating and encircling indentations.

Figure 2. Scanning electronmicrograph of arthroconidia of *T. mentagrophytes* adhering singly, or in pairs and in clusters to the margin of a corneocyte, 6 h incubation at 37°C.

the surface area and nutrients available for growth of dermatophytes on stratum corneum. Fourthly, in the pathological condition of palmoplantar hyperkeratosis, which is characterised by a dramtic increase in the thickness of the stratum corneum, there is frequent occurrence of dermatophytosis.

Source of infection

Experimental human infection has been developed using different morphological forms of dermatophytes on different areas[1]. Infection has been induced with macroconidia, microconidia, arthrospores from natural infection of hair, fragments of agar cultures, and dermatophyte-infected skin scales. From these studies it can be concluded that all of the different morphological forms of dermatophytes have the potential to cause human infection, but arthroconidia, because of their *in vivo* formation and shedding from lesions, are likely to be the forms involved in the spread of infection. Within lesions dermatophytes exist also as hyphal elements and these too are shed, and since direct contact with a lesion is considered to be one mode by which dermatophytes are spread, it seems probable that hyphae also play an important role in the spread of dermatophytosis. In the direct mode of dermatophyte spread, i.e. contact with exfoliated infected material, the role of arthrospores is substantiated by the fact that besides being spores, i.e. non-vegetative and thus having no exogenous nutritional requirements, they are resistant to adverse conditions. They can also be produced in large numbers, especially when hair is involved where myriads of them have been observed. The role of hyphal elements of dermatophytes in the indirect mode of spread of infection can be considered to be dependent on their ability to survive in the environment.

Since arthroconidia are produced by fragmentation of hyphae it is suggested that these fungal cells are the most suitable of the dermatophyte spores for the growth of dermatophytes in the stratum corneum, unlike micro- or macroconidia which are formed laterally and apically on hyphae, thereby requiring a space for their expansile type of development (Figure 1). This mode of growth will help dermatophytes escape the epidermal exfoliative capacity and at the same time produce contagious propagules. Furthermore, it has been shown that conditions of reduced humidity enhance separation of arthrospores from each other, a condition that would approximate to that found in exfoliated skin scales.

Experimental models of dermatophyte and non-dermatophyte invasion of keratinous substrates

We have developed a number of experimental models to approximate the natural environment of dermatophytes using arthroconidia as the most relevant infectious particle. Conidia and hyphal fragments have been used to study keratin digestion by non-dermatophytes.

Dermatophyte adhesion to the stratum corneum

Adherence of microorganisms to host tissues is an important step in the establishment of disease. It is how host surfaces are colonised. *Candida albicans* for example, has been shown to adhere to mucosal cells and corneocytes. Unlike candidosis, dermatophytosis is acquired from an exogenous source because dermatophytes are not part of the normal skin flora. Thus initial contact between arthroconidia and stratum corneum would be seen to be an important event in the establishment and initiation of the infection of the stratum corneum [2]. Simple models of dermatophyte adherence include suspension assays using separated corneocytes and fungal cells and stripped stratum corneum have shown that adherence of

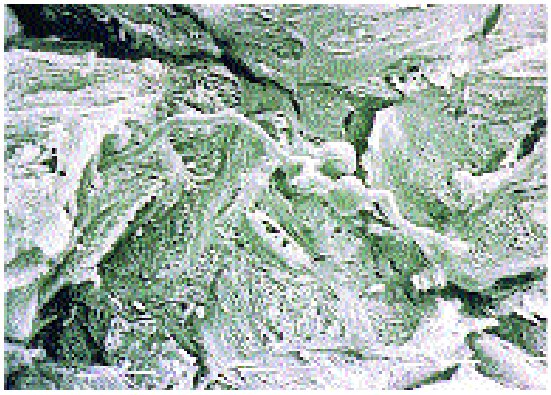

Figure 3. Scanning electronmicrograph of arthrospore germination in the presence of corneocytes, 21 h incubation at 37°C. Germ tubes appear to be penetrating the stratum corneum.

arthroconidia takes place and is dependent on close approximation between the walls of the two cells with the adherence site filled with floccular material. The nature and origin of this material, i.e., that from the arthrospore wall, the stratum corneum or both, along with the mechanisms involved, require further investigation. Another point which needs elucidating is the influence of sebum on arthrospore adherence to corneocytes. In our own studies corneocytes from palm and sole were used in adherence assays and both sites are devoid of sebaceous glands.

Using the corneocyte model it was found that adherence of arthroconidia of *T. mentagrophytes* was time dependent and reached a maximum after 6 h, by which time germination had started [3]. Significant differences were seen between the strains in their adherence to corneocytes from the palm. Not all corneocytes had adherent arthroconidia, although there was a time-dependent increase in the numbers of corneocytes with adherent fungal cells. By scanning and transmission electron microscopy it was seen that there was a loose association between arthroconidia and corneocytes with no apparent damage to the corneocyte membrane (Figures 4 and 5). Adherence of germlings of *T. mentagrophytes* to corneocytes appeared to be mediated by germling outer cell wall fibrils. Hyphal branches and secondary germlings were observed to enhance the attachment of the parent hypha to adjacent corneocytes.

As an alternative to animal models or separated corneocytes for investigating pathogenic mechanisms adhesive tapes and cyanoacrylate contact cement have been used to sample lesions of dermatophytosis and uninvolved skin. Stripping the stratum corneum by cyanoacylate adhesives has been termed skin surface biopsy [4] and has been used in many investigations of the biology of infection of the stratum corneum. Since the stratum corneum is composed of dead cells in the process of exfoliation, and dermatophytes in lesions remain confined to it, the use of stripped sheets of stratum corneum to study the biology of dermatophytes is appropriate. Furthermore, the model allows the structural integrity of the stratum corneum to be maintained under a number of environmental conditions.

Using a stripped stratum corneum model where the upper layers (about 5 corneocytes thick) of the skin were removed from uninvolved skin we found that arthroconidia of *T. mentagrophytes* increased in size and started germination by 4 h at 37°C [5]. Germ tubes originated from a point on the arthroconidium surface mid-way between the points of attachment to adjacent conidia. With further incubation germination increased and germ tubes extended across the stratum corneum. Histological staining of transverse sections of infected stratum corneum showed hyphae penetrating longitudinally and perpendicularly through the thickness of the stratum corneum. By 7 days' incubation hyphae started to form arthroconidia thereby completing the vegetative growth cycle of the fungus (Figure 6). Scanning electron microscopy revealed penetration of corneocytes by germ tubes resulting in damage to the corneocyte surface. The horizontal extension of hyphae in this model is probably comparable to the clinically observable sign of peripheral expansion of lesions of dermatophytosis. Dermatophytes can be cultured from skin around the margin of lesions for a distance of up to 6 cm [6]. The penetration of germ tubes into deeper layers of the stratum corneum has also been reported in experimentally induced dermatophytosis in guinea pigs and was found to take place to a greater extent by a zoophilic strain of *T. mentagrophytes* than seen with an anthropophilic strain of *T. mentagrophytes.*

Figure 4. Scanning electronmicrograph of arthroconidia of *T. mentagrophytes* adhering to stripped stratum corneum from leg and incubated at 37°C for 6h. Germ tube tips are in close contact with the stratum corneum.

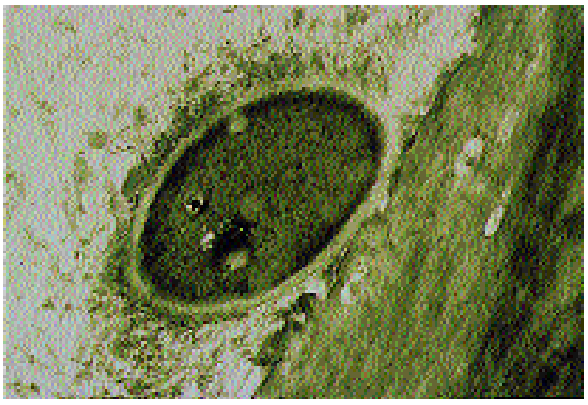

Figure 5. Transmission electronmicrograph of an arthroconidium of *T. mentagrophytes* adhering to the margin of a corneocyte. There appears to be some digestion of the corneocyte membrane.

Dermatophyte adhesion and invasion of living skin equivalents

As stated above, there are few satisfactory *in vitro* experimental model systems in which to study the early events in the colonisation of skin by the arthroconidial parasitic form of dermatophytic fungi and their response to antifungal agents. The living skin equivalent has a very close resemblance to the normal tissue [7]. Because of its use in pharmacological studies, the living skin equivalent was considered appropriate to assess the mode of action of terbinafine [8].

Arthroconidia were inoculated onto skin-equivalents and incubated at 28°C for seven days. Fungal growth was assessed by light and scanning electron microscopy. On gross examination fungal growth was seen on the surface of the models around four days of incubation. When therapeutic concentrations of terbinafine were applied to the surface of the model or incorporated into the medium, no growth was seen and a clear surface was visible. On light microscopy control H & E sections showed a well-stratified epidermis, including a well developed stratum corneum. Sections fixed around 72 h showed evidence of arthroconidial germination, with the fungal elements growing on the stratum corneum, and with evidence of horizontal and perpendicular penetration on the stratum corneum by the germ tubes. There was no penetration below the stratum corneum at this stage. Sections fixed

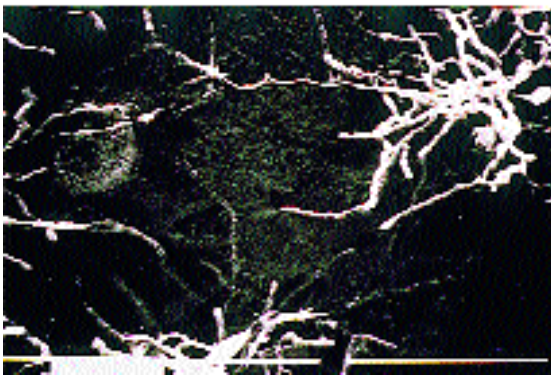

Figure 6. Scanning electronmicrograph of arthrospores of *T. mentagrophytes* inoculated onto a stripped stratum corneum sheet and incubated for 7 days at 37°C. Fungal hyphae can be seen to have disarticulated into arthrospores with some segments of hyphae remaining unfragmented.

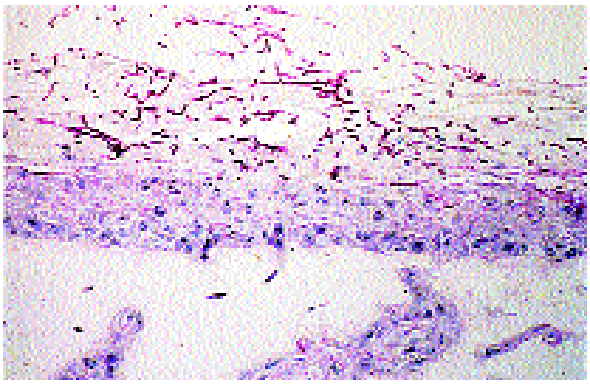

Figure 7. Histopathological section of a living skin equivalent showing the growth of *T. mentagrophytes* restricted to the stratum corneum and epidermis and not penetrating into the dermis. Six days incubation at 28°C.

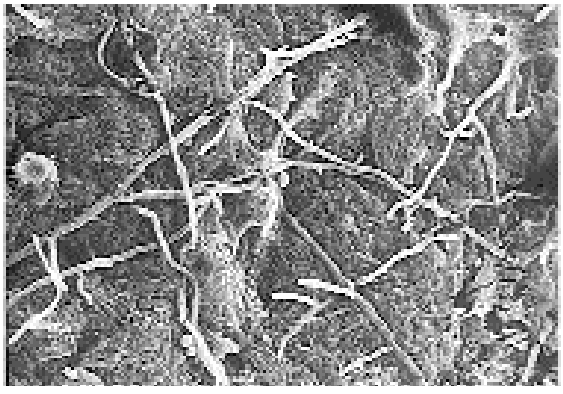

Figure 8. Scanning electronmicrograph of penetration of contracted collagen lattices by hyphae of *T. mentagrophytes*.

around six days showed extrensive growth of fungal elements on the stratum corneum and penetration of the epidermis, replacing most of it with mycelium (Figure 7). Fungal hyphae were observed growing throughout the epidermis and lying on the upper dermis as a result of penetration from the epidermis.

Where terbinafine had been incorporated in the medium, growth inhibition was seen in the range 0.01 – 1.0 mg/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. At 1 mg/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.

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 fibres was observed. Adherence to keratinocytes was particularly prominent, with a depression formed where arthroconidial binding had occurred. Some arthrocondia 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 48 h, with the arthroconidia varying in the size range 8-10 µm. Little germination of arthroconidia was seen at this stage. By six days, germination of arthroconidia had taken place, with hyphal extension occurring on the surface of the skin equivalent and side branches appearing with a well formed mycelium (Figure 8). Penetration of the model was evident where germlings were growing through pores and cavities. Germlings were seen to grow very well on collagen matrix and were observed penetrating pores in the collagen lattices. Segmentation and arthroconida was evident in the hyphae.

Invasion of non-dermatophytic moulds on living skin equivalents

Using the same living skin equivalent model we studied the ability of non-dermatophytic moulds that are occasionally associated with onychomycosis as primary pathogens, namely, *Scopulariopsis*, *Fusarium* and *Acremonium*. All three moulds germinated on the surface of the skin equivalent as assessed by gross examination. In histopatholgical sections it was seen that *Scopulariopsis* was almost entirely confined to the stratum corneum with very little penetration into the epidermis (Figure 9). In contrast, *Fusarium* and *Acremonium* were seen to have invaded the full thickness of the epidermis with some degree of invasion of the dermal layer (Figures 10 and 11).

Dermatophyte adhesion and invasion of nail

The nail is predisposed to attack by many microorganisms, including fungi, some of which invade the living tissus of the nail bed and nail fold; others invade the nail plate itself. To understand the ability of fungi to invade the human nail it is important to appreciate the various anatomincal regions of the nail plate. In the free part of the human nail there is stratification of the horny lamellae such that a dorsal nail, an intermediate nail and finally a ventral nail can be distinguished on histology. The hyponychial keratin of the ventral nail consists of polyhedral non-nucleated cells, arranged loosely and irregularly. This situation corresponds somewhat to that found in the desquammation process of the upper layers of the stratum corneum. As in the skin, the loose layers in the nail are preferred sites for fungal infection, especially as it possible for these organisms to migrate directly from the surrounding skin into the adjacent region. In the free part of the nail, a layer is firmly attached to the underside which constitutes a differentiation product of the hypony-

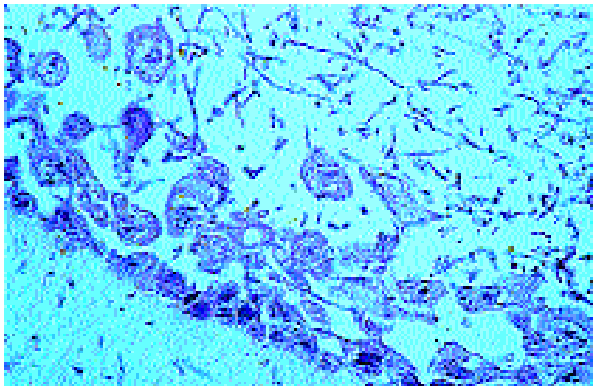

Figure 9. Histopathological section of *Scopulariopsis brevicaulis* infecting a living skin equivalent.

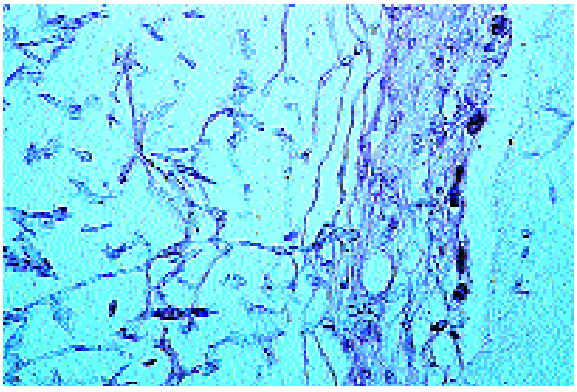

Figure 10. Histopathological section of *Fusarium* invading a living skin equivalent.

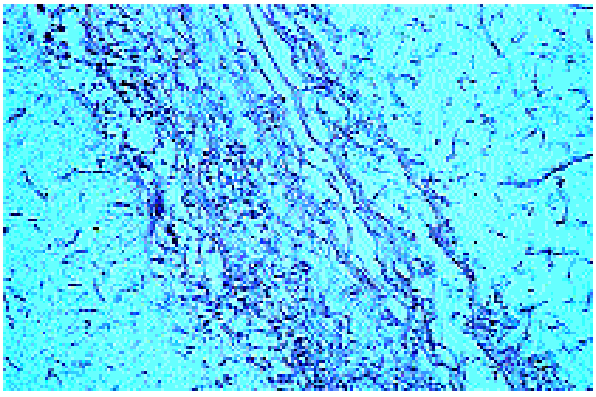

Figure 11. Histopathological section of *Acremonium* invading a living skin equivalent.

chium and may hypertrophy in many disease processes. The process of keratinisation over the stratum granulosum as exhibited in the skin is replaced in nail formation by the process of onychisation, due to the functionally different duties of the horny layer of the skin and the keratin of the nail.

The ability of dermatophyte fungi to utilise the keratin of the nail plate as a growth substrate is well characterised, but the status of primary pathogens of non-dermatophytic moulds and yeasts, such as *Scopulariopsis brevicaulis, Acremonium* spp. or *C. albicans*, is contentious.

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. There are only a few i*n vitro* studies examining the process of nail invasion by dermatophytes. Even less is known regarding the ability of non-dermatophyte moulds to invade the nail plate. It is evident that all previous studies on the ability of dermatophytes to invade nails *in vitro* have used various culture media to promote growth on nail keratin.

There are few satisfactory experimental models for the study of the ability of fungi to colonise and invade the nail plate. Furthermore, little is known regarding the activity of antifungal agents in nails and the morphological changes in dermatophytes and non-dermatophytic moulds after exposure to antifungal drugs in the nail plate.

We developed an *in vitro* nail model comprising of arthroconidia of *T. mentagrophytes* and fragments of finger nails and toe nails [9]. Arthroconidia were inoculated on the ventral surface of the nails. After 6 h, adherence and germination of arthroconidia was observed. By 16 h, small germ tubes with side branches were evident. At about 24 h, micro-colonies had become established. At 48 h, mycelium formed, and at about 72 h most of the nail fragment was covered with fungal growth (Figures 12 and 13). Nail penetration occurred from the ventral surface through the intercellular spaces, and with longer incubation all three layers were invaded by arthroconidia growing through the channels (Figure 14). Nail invasion occurred in the absence of added nutrients. Dermatophyte

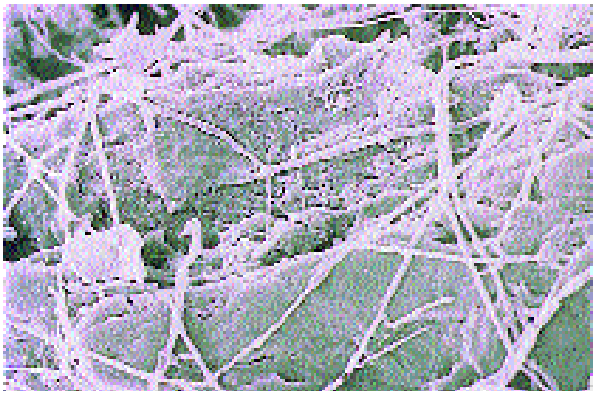

Figure 12. Scanning electron micrograph of a nail fragment covered with a mycelial mesh of *Trichophyton mentagrophytes* after 72 h incubation.

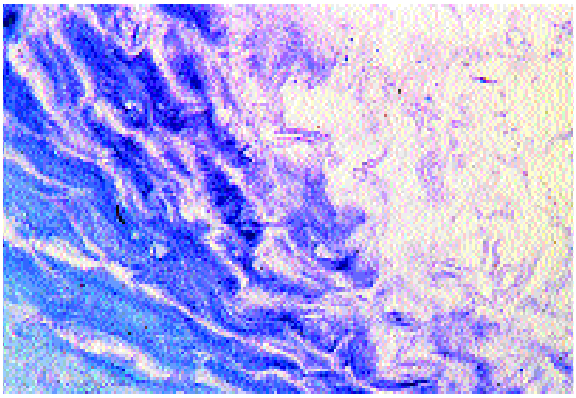

Figure 13. Toluidine blue-stained section of a nail fragment infected with *T. mentagrophytes*.

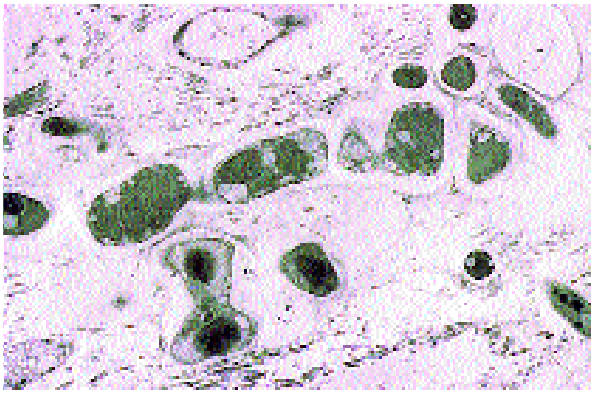

Figure 14. Transmission electronmicrograph of *T. mentagrophytes* arthroconida formed from penetrating hyphae in nail.

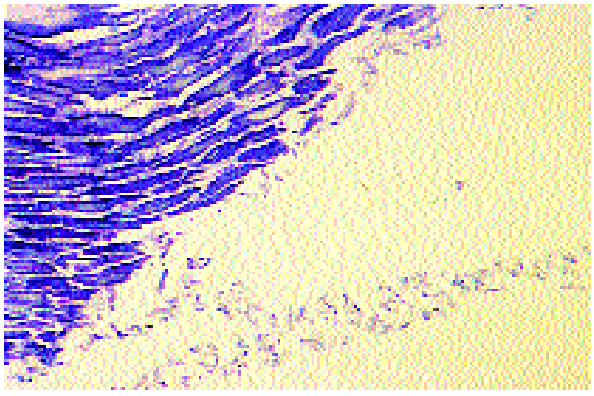

Figure 15. Toluidine blue-stained section of surface growth of *T. mentagrophytes* on a nail fragment previously exposed to itraconazole.

fungi appeared to invade the nail by a combination of mechanical and chemical factors. In this model it was interesting to note that there were similarities betweeen the growth of fungus on the nails and on the stratum corneum. The findings are similar to those obtained in investigations of patients with mycotic nails, except that all three layers were invaded by the fungus, and this rarely occurs *in vivo*. Previous models of nail invasion have employed nutrients to enhance nail penetration by dermatophyte fungi, but in our model nail infection was established in the absence of these factors.

We have used this model to determine the bioavailability of terbinafine [10]. It was found that preexposure of nail fragments to terbinafine concentrations (0.001 to 10 mg/l) inhibited fungal growth and acted as a barrier to dermatophyte invasion. Damaged arthroconidia and distorted hyphae on the surface of nail fragments were observed. Similar results were seen when itraconazole was used (Figure 15). The model provided an alternative system for studying the activity of antifungal agents in nail and was useful to demonstrate the morphological changes in dermatophyte fungi after exposure to terbinafine.

Pathogenic potential of non-dermatophytes in invading the human nail

The status as primary pathogens of non-dermatophytic moulds and yeasts, such as *Scopulariopsis brevicaulis*, *Acremonium* species or *C. albicans*, is contentious. Very little is known regarding the ability of non-dermatophytic moulds to invade the nail plate. Using a nail model as described above where there were no additional nutrients incorporated into the model the growth of *S. brevicaulis*, *Acremonium* sp., *Fusarium* sp. and *Aspergillus versicolor* was first visible on the surface of nail fragments from healthy subjects at about 4 days [11]. By seven days, the nail fragments were totally covered by fungal mycelia. Nail fragments digested with potassium hydroxide showed hyphae growing on the surface. Toluidine-blue-stained transverse sections showed that *Fusarium* formed distinct channels throughout the thickness of the nail plate. *A. versicolor* grew only over the surface of the nail. Very little growth of *S. brevicaulis* was seen apart from a few conidia. *Acremonium* sp. grew on the surface of the nail with some invasion of the outer aspect of the fragment. In some respects the pattern of invasion of these non-dermatophytic moulds reflects their ability to invade through the stratum corneum and epidermis of living skin equivalents (see above).

This series of experiments has shown that non-dermatophytic moulds vary in their ability to grow on and invade the nail plate. Others have shown that keratinised substrata can be colonised by common moulds with no known keratinolytic properties [12]. Invasion of nail was by means of specialised and non-specialised hyphal forms. Similar appearances were seen for hair and callous keratin. The means of invasion varied according to the physical nature of the substratum. Those materials with a stratified and resistant physical structure induced the formation of mycelial fronds and boring hyphae. Digestion by the fungi in substrata containing hard keratin was limited to the non-keratinised intercellular structures. Although in our study there appeared to be major differences in the ability of moulds to invade the hard structure of the nail plate, there was no obvious parallel with the predominant moulds, i.e. *S. brevicaulis* and *Acremonium* associated with onychomycosis. It appears that our model system is of limited applicability to the complex situation found in nail infection by fungi in man. However, our observations made with this model do support the contention that some moulds have the inherent ability to utilise keratin, perhaps in a limited fashion, but that individual variations of the nail plate environment could modulate the pathogenicity of fungal elements adhering to the nail surface.

Dermatophyte adhesion and invasion of the hair follicle

Affected hair tissues from cases of *tinea capitis* have been studied using light microscopy, scanning and transmission electron microscopy. However, ultrastructural findings of the parasitic form of *T. mentagrophytes* in hair tissue have been inconclusive and the studies have not used intact hair follicles. The pathological changes of the affected hair structure are poorly understood. The stages by which detached hairs are attacked by keratinophilic fungi are: (1) cuticle lifting, (2) cortical erosion, (3) production of penetrating organs, and (4) colonisation of the medulla. 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 appratus are significantly different between fungi. Experimental studies of hair penetration by dermatophytes are few and have been restricted to cut or plucked hair, and arthroconidia have not been previously used in experimental studies of hair infection. There have not been any satisfactory experimental

Figure 16. Scanning electron micrograph of a plucked hair showing growth of fungal hyphae on the hair shaft after incubation for 40 h at 28°C.

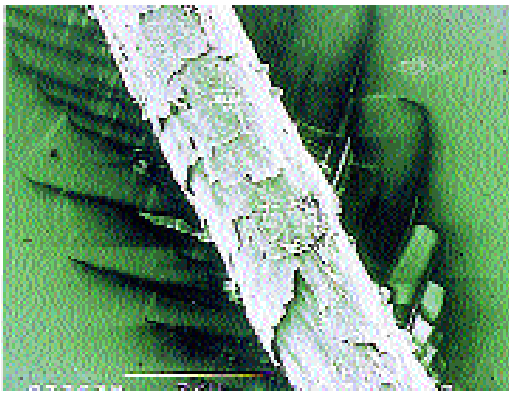

Figure 17. Scanning electron micrograph of a plucked hair showing growth of fungal hyphae on the hair shaft after incubation for 40 h at 28°C. Note the flaps of raised cuticle.

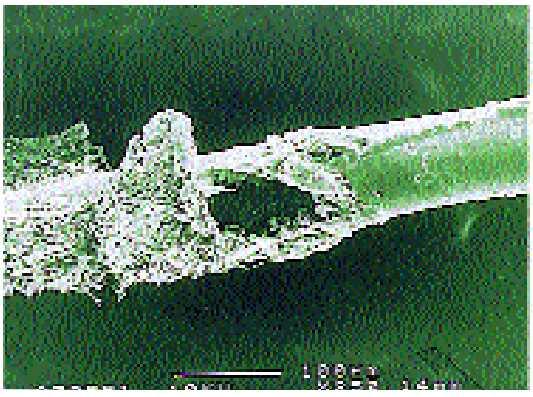

Figure 18. Scanning electron micrograph of complete destruction of a hair shaft by *T. mentagrophytes*.

studies for detailed study of the invasion of hair follicles by dermatophyte fungi. We developed a novel in vitro model for the study of hair invasion by *T. mentagrophytes* [13]. Hair was obtained by microdissection and plucking. Following inoculation of the hair follicle with arthroconidia, growth of the fungus was seen on the hair and within the follicle (Figure 16). Growth was observed to begin at the shaft end and to extend along the hair shaft towards the bulb area. In follicles maintained in organ culture the inner root sheath in particular was invaded by *T. mentagrophytes* and provided a good substrate for fungal growth. Initially, the cuticle formed a barrier to fungal penetration of the hair. After incubation for four days, germlings of *T. mentagrophytes* were seen to penetrate under the cuticle and in between the layers of cuticular cells to invade the cortex (Figure 17). There was no evidence of intracellular growth; fungal elements were seen intercellularly (Figure 18). There were similarities between the findings in this study of the process of hair invasion by dermatophyte fungi and that in the natural disease. Based on the findings of our model it appears that the process of hair invasion by dermatophytes is a combination of both mechanical and chemical forces, one augmenting the other.

In a subsequent study we used the hair follicle model to determine the mode of action of terbinafine on the growth of *T. mentragrophytes* [14]. Arthroconidia were inoculated onto hair follicles obtained by either micro-dissection or plucking that had been exposed to therapeutic concentrations of terbinafine. Where hair follicles were exposed to low concentrations of terbinafine (0.01 – 1.0 mg/l), inhibition of growth was seen and morphological changes were observed in the arthroconidia and germlings. Our model seems to provide a system for studying the process of hair invasion by dermatophyte fungi and the effects of antifungal drugs in hair follicles and their mode of action in *tinea capitis.*

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