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Colonization and penetration of the stratum corneum by dermatophyte fungi

by

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submitted in fulfilment of the requirment

for the degree of Doctor of Philosophy

in the Department of Dermatology,

The University of Glasgow.

April,1990

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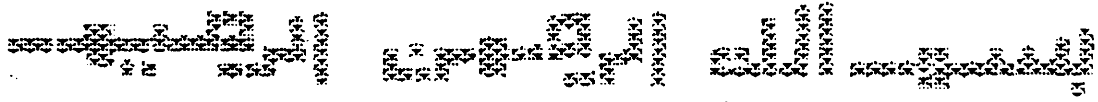


(In the name of Allah, most Gracious most Merciful)

DEDICATED TO MY PARENTS, BROTHERS, SISTERS, AND WIFE

SUMMARY

The colonization and penetration of stratum corneum by dermatophyte fungi were investigated employing arthrospores of three strains, two of Trichophyton mentagrophytes and one of Trichophyton interdigitale. The adherence of arthrospores to corneocytes from palm and sole and germination in suspensions of corneocytes from the same body areas were determined. The growth of arthrospores on stripped sheets of stratum corneum from different body areas, namely, sole, leg, groin, abdomen, back, cheek, forearm, and palm, was also studied. The ultrastructure of corneocyte and stratum corneum - dermatophyte relationship was investigated by scanning and transmission electronmicroscopy. Scale from cases of tinea pedis was also investigated ultrastructurally. The resistance of arthrospores to ordinary environmental and desiccated conditions in the presence and absence of corneocytes was studied and the type of dormancy expressed by arthrospores was investigated. The effect of exposing arthrospores to distilled water for 24 hr on their germination in corneocyte suspensions and on stratum corneum was studied.

The possibility of using corneocytes as a model for assessing antifungal activity of drugs against dermatophytes was explored. Two phases of investigation were conducted; phase I consisted of arthrospore germination in corneocyte suspensions in the presence of antifungal drugs and phase II consisted of firstly inducing arthrospore germination in corneocyte suspensions then adding antifungal drugs.

Arthrospore formation was studied under various cultural conditions; temperature of 37°C, elimination of glucose from growth media, presence of amphotericin-B in growth media and increased tension of carbon dioxide. With the exception of amphotericin-B arthrospore formation occurred and was especially abundant with carbon dioxide. Ultrastucturally, the arthrospores appeared mainly round and were surrounded by a thick wall of which the outer aspect appeared fibrillar. Disarticulated arthrospores were seen connected by fibrils likely to be remnants of the original hyphal wall.

Adherence of arthrospores to corneocytes occurred and showed a time dependent increase up to 6 hr by which time arthrospores had started to germinate. The adherence of arthrospores to corneocytes was verified by scanning and transmission electronmicroscopy. A floccular material bridged the closely opposed walls of arthrospores and corneocytes. The surface of corneocytes appeared convoluted and arthrospores adhered to more than a convolution of the surface thereby conferring a firmer attachment.

Germination of arthrospores in corneocyte suspensions occurred and showed also a time dependent increase up to 16 hr by which time long branched germ tubes had developed. Both corneocyte-adherent and nonadherent arthrospores had germinated and sometimes the arthrospores had multigerminated. Germ tubes adhered to corneocytes and penetrated them. Corneocytes penetrated by germ tubes appeared ragged.

Growth of arthrospores on stratum corneum started by germination at 4 hr which increased with time up to 16 hr by which time long branched germ tubes had developed. By 24 hr distinct fungal microcolonies, consisting of arthrospores which had multigerminated and germ tubes which had multibranched, developed. Germ tubes penetrated transversely and through the thickness of the stratum corneum. By 7 days masses of hyphae had broken down into arthrospores but some hyphal branches and terminal segments remained aseptate. Ultrastructurally, small germ tubes were either appossed to the surface of corneocytes, attached to the margin of corneocytes, insinuated between corneocytes or appeared to be inserted in them. Growing germ tubes either extended over the surfaces of corneocytes, penetrated or appeared buried in them producing ridges. By 7 days the corneocytes appeared disintegrated. In the scale from cases of tinea pedis, fungal elements were located within the keratin and surrounded by electronlucent zones. Germination of arthrospores had not occurred on the stratum corneum in the absence of moisture or oxygen or at 45°C and 4°C. On the outer layers of stratum corneum arthrospore germination was statistically not different from inner layers. In the absence of stratum corneum arthrospore germination was minimal, 0.00-3.44±1.06 after 16 hr, and the germ tubes were short, thin and only faintly stained with Periodic acid-Schiff.

Exposing the arthrospores to distilled water at 28°C for 24 hr prior to their inclusion in corneocyte suspensions or inoculation onto stratum corneum increased germination.

Under conditions not suitable for germination the arthrospores expressed an exogenous type of dormancy provided that these conditions were not lethal. Conditions under which the arthrospores expressed an exogenous type of dormancy were found to be: absence of moisture or nutrient and 4°C. A condition found to be lethal was 45°C.Survival of arthrospores exposed for 6 days to ordinary environmental and desiccated conditions was greater in the presence of corneocytes than in their absence, the difference reached significant levels under environmental conditions, P values were less than 0.05 and 0.01.

In phase I of the assessment of antifungal activity of drugs, griseofulvin and clotrimazole against dermatophytes using the corneocyte model, arthrospore germination was significantly reduced, P value was exceedingly less than 0.001. In phase II it was arrested at levels statistically not different from those of the initial incubation without antifungal drugs but less than those of the control. In summary this study contributed the following findings with regard to the pathogenesis and epidemiology of dermatophytosis and in vitro testing of antifungal drugs:-

A - Pathogenesis:

1 - Arthrospore fomation in vitro occurs under conditions which resemble those seen biologically in the skin.

2 - Adherence of arthrospores to corneocytes occurs but germination and penetration of stratum corneum by germ tubes is more important in the establishment of infection. This is because if a corneocyte-adhering arthrospore fails to germinate before this particular corneocyte is shed from the skin by the epidermal exfoliative process it is likely to be shed with it. Also the aforementioned arrangment of small germ tubes on the stratum corneum is apt to secure the position of the whole fungal structure (arthrospore with its germ tube) during the initial stage in the development of infection.

3 - In colonizing the stratum corneum dermatophytes grow transversely in and through its thickness and penetrate individual corneocytes.

4 - In hyphal masses breaking down into arthrospores the presence of aseptate hyphal branches and terminal segments may reflect the continuation of growth in these branches and terminal segments. This combined mode of growth and arthrospore formation is more likely to help the dermatophyte to escape the epidermal shedding while at the same time produces its reproductive and infective particles.

5 - Adherence, germination and germ tube elongation can be considered as pathogenetic mechanisms of dermatophytes.

B - Epidemiology:

1 - The increased germination of arthrospores which were hydrated initially implies that, arthrospores shed into moist areas such as the floor of sport centres and communal

bathing facilities or wet items such as used towels are likely to have better chances of causing infection in new hosts.

2 - Under conditions not suitable for germination but not lethal the arthrospores survive in an exogenous type of dormancy.

3 - Arthrospores acquire some form of protection from the stratum corneum which enable them to survive longer outside the skin.

C - In vitro assessment of antifungal drugs:

The corneocytes provide a simple, inexpensive, and rapid model for the assessment of antifungal drugs against dermatophytes.

Chapter 1

General Introduction

1.1 Dermatophytes:-

Dermatophytes are a group of closely related filamentous fungi which infect superficial cornified tissues (stratum corneum of skin, hair and nail) producing the disease variously known as dermatophytosis, tinea or ringworm.

The dermatophytes grow well in artificial media containing organic sources of nitrogen, the commonly used ones are malt agar and glucose peptone agar supplemented with antibiotics to combat bacterial and saprophytic mould contamination. From the point of inoculation the filaments, hyphae, grow and form lateral branches periodically behind their apices as they elongate resulting in a network which make up the mycelium. On the sides and tips of hyphae spores develop, these are the small and unicellular microconidia, and the large and multicellular macroconidia. At times, round and thick-walled structures called chlamydospores are produced either singly or in chains (Simon and Galgoczy, 1986). Depending on the appearance of the macroconidia, dermatophytes are divided into three genera, Microsporum, Trichophyton and Epidermophyton. In the genus Microsporum the macroconidia are spindle shaped, their walls are thick and toughened with projections and transversed with up to 15 cross walls; in the genus Trichophyton the macroconidia are club or cylindrical in shape, their walls are thin and smooth with up to eight cross walls; and in the genus Epidermophyton the macroconidia are club shaped or oval and their walls are thin and smooth with only a few cross walls. Microconidia are not seen in the genus Epidermophyton.

In a dermatophytic lesion a different type of spore is seen, the arthrospore. Arthrospores are produced by a cross-walling (septation) of hyphae with a subsequent segregation of the resulting compartments. Their formation is typical of the dermatophytes when parasitizing the superficial cornified tissues.

A distinct type of spore formation is seen when two different "mating" types of a dermatophyte designated as + and - or "A and a" come in contact with each other in a suitable, but hostile, substrate lacking readily assimilable nutrients such as hair mixed with soil. Under such conditions the hyphae of the two dermatophytes anastomose, their nuclei fuse and undergo meiosis to restore the haploid state of the fungus, and with subsequent mitotic divisions eight spores called ascospores are formed, half of the plus and half of the minus mating type. The ascospores are contained in a thin walled envelope called an ascus. Asci are produced in groups enmeshed by

networks of modified hyphae called peridia; the whole structure is called a cleistothecium. Because opposite mating types are required for ascospore formation, the dermatophytes are classed as heterothallic fungi, and the ascospores as sexual spores. The corresponding life cycle of dermatophytes is described as sexual, perfect or telemorphic. On the other hand the formation of microconidia, macroconidia, chlamydospores and arthrospores is independent on the union of any dermatophytic forms, and so they are described as asexual spores and the corresponding life cycle of dermatophytes as asexual, imperfect or anamorphic. Knowing the perfect stages of some of the dermatophytes has allowed their reclassification from the phylum Fungi Imperfecti (Deutromycota) to the phylum Ascomycota where they are represented in the family Gymnoascaceae by the genus Arthroderma and the genus Nannizia which correspond to the imperfect stages of Trichophyton and Microsporum respectively (Ajello, 1977). However, recently Weitzman et al., 1986, evaluated the morphological characterization used to define the genera Arthroderma and Nannizzia and reported that these taxa are congeneric, and owing to priority they considered Nannizzia as a later synonym of Arthroderma.

Perfect stages have been described for almost all the geophilic dermatophytes but only for a few zoophilic species and none for the anthropophilic dermatophytes (Hasegawa, 1988). Of the medically important dermatophytes three have known perfect stages, Arthroderma benhamiae and Arthroderma vanbreuseghemii for Trichophyton mentagrophytes complex, Arthroderma gypseum and Arthroderma incurvatum for Microsporum gypseum and Arthroderma otae for Microsporum canis.

The dermatophytes are of world wide occurrence and although there are some geographical differences in their distribution these differences are gradually narrowing as a result of migration and mixing of populations (Ajello 1960, Badillet, 1988).

1.2 Dermatophytosis:-

Dermatophytosis, tinea, involves the stratum corneum of the skin, hair and nails. Clinically there are eight regional types; tinea capitis, tinea faciei, tinea barbae, tinea corporis, tinea cruris, tinea manum, tinea pedis and tinea unguium. The clinical picture of a tinea lesion is thus dependent, largely, on its location on the body, but basically the lesion is scaly, erythematovesicular with peripheral extension and central clearing.

Destruction and disorganization of hair and nails occur at various degrees of intensity whenever they are involved.
The zoophilic and geophilic infections usually run a more inflammatory and shorter course than the anthropophlic.The dermatophytoses are common and of worldwide distribution, affecting both sexes and all age groups and can occur in an epidemic form.
Although the dermatophytes grow only superficially in the skin, they can induce a number of reactions in the host; local histopathological changes, distant cutaneous eruptions and systemic immunological responses. The local histopathological changes take the picture of acute, subacute or chronic dermatitis depending on the degree of reaction of the skin to the presence of the dermatophyte (Lever and Schaumberg-Lever, 1983).
The distant cutaneous eruptions are the ids (dermatophytids) which may appear at the height of an inflammatory flare in a lesion and take up different clinical forms, of which the well recognised is the eczematoid on the hands and sides of the fingers in an adult with tinea pedis. They are negative for the presence of dermatophytes and usually resolve with the clearance of the primary infections.
The systemic immunological responses induced by the dermatophytes are both antibody and cell mediated. While the antibody response occurs in the inflammatory and non-inflammatory types of infection (Grappel, Blank and Bishop, 1971;1972) the cell mediated response as indicated by delayed type hypersensitivity occurs more readily in the inflammatory type of infection (Jones, 1980).

1:3 Skin

The skin is the largest organ of the body. It is composed of an outer epithelial layer, the epidermis, a middle connective tissue matrix, the dermis and an inner adipose tissue the hypodermis.
The epidermis is made up of cells called keratinocytes. These cells are arranged in several strata, the deepest one is the stratum basale which forms the germinative compartment of the epidermis. From stratum basale cells migrate upward to form first cells of the stratum spinosum, then cells of stratum granulosum and finally cells of the outermost stratum of the epidermis, the stratum corneum, from which cells (corneocytes) are continuously shed into the exterior. During their upward movement keratinocytes undergo gradually the generally known process of

keratinization (cornification). Cornification involves not only synthesis of the principle fibrous protein, keratin inside the keratinocytes but other distinct cellular changes (Elias, 1987). These changes include, one, generation of neutral lipid-enriched intercellular spaces by the secretion of Odland bodies. Two, synthesis of stratum corneum basic histidine rich protein (fillagrin), which constitutes the major portion of the keratohyaline granules of the stratum granulosum. Three, formation of a highly cross-linked insoluble peripheral envelope of corneocytes which is composed of two or more precursor proteins, including involucrinand keratolinin. In addition to these changes cells passing to the stratum corneum become flattened and their nuclei and cytoplasmic organelles become disintegrated.
Epidermal cells are attached to one another by the complex lamellated structures, the desmosomes, which break and reform to allow upward movement of cells. They also serve as anchoring plates for keratin filaments. Beside keratinocytes, the major cellular components of the epidermis are three types of dendritic cells: melanocytes, melanin producing cells; Langerhans' cells, immunologically functioning cells (Sauders, 1983, Wolff and Stingl, 1983) and indeterminate cells thought to be related to Langerhans' cells (Chu et al.,1982) in addition to Merkel's cells which are thought to have a neuroendocrine function (Marks, Knight and Laidler, 1986).The epidermis contains no blood vessels, capillaries in upper dermis transudate nutrients into it.
Through the basement membrane the epidermis is connected to the dermis. In the dermis there are connective tissue fibres (collagen, elastin and reticulin), fibroblasts and amorphous ground substance. Located in the dermis are blood and lymphatics vessels, nerve and nerve endings, arrector pili muscles and adnexal structures (hair follicles, sebaceous, apocrine and eccrine sweat glands). The eccrine sweat glands are distributed over the whole body surface but are especially abundant on the palms and soles. The aprocrine glands are present mainly on the axillary and anogenital areas and the sebaceous glands are absent on the palms and soles.

1:4 Stratum Corneum:-

The stratum corneum is the outermost layer of the skin. It is built up of highly specialized but dead cells (devoid of nuclei and cytoplasmic organelles). These cells, called corneocytes are continuously shed into the exterior from the surface of the stratum corneum in a process well balanced

with the migration of cells from beneath into the stratum corneum. This process is known as exfoliation or desquamation. The time required for the renewal of stratum corneum is about two weeks (Jansen, Hojyo-Tomoko and Kligman, 1974).

The corneocytes are the largest cells of the epidermis. They are flattened and polyhedral in shape measuring about 0.8μm in depth and 30μm in width (Baker, 1987). They are joined by modified desmosomes and their surfaces are thrown into convolutions interlocking with cells above and below with their lateral margins overlapped. They are arranged in layers which are about 12-20 in number over most of the body surfaces but are less in some areas e.g. scrotum, more in others, e.g. back of hands and most on palms and soles where the stratum corneum is almost 300-400μm in thickness (Baker, 1987). With the notable exception of palms and soles, where the stratum corneum is thick and overlapping of corneocytes is most evident, corneocytes are often arranged in vertical stacks. (Hume and Potten, 1983).

Currently, the stratum corneum is viewed as a two compartment system of protein enriched cells embedded in a lipid-laden intercellular space (Elias, 1987). Keratin makes up to 80% of the cellular protein (Green, 1979). It is arranged in filaments anchored to desmosomal plates. The two compartments are separated by highly resistant corneocyte membranes which have defied all attempts to solublize and analyse them (Blank, 1987).

Sweat and sebum contribute to the elements of stratum corneum. Sweat, in addition to electrolytes, contains a number of various amino acids and glucose (Boysen et al., 1984) and sebum is a complex mixture of lipids.The overall composition of stratum corneum is approximately 65% keratin, 20% remnants of nuclear and cytoplasmic components, 7-9% lipid and 5% cell membranes (Matoltsy, 1958).

Crude soft horn production was found to be higher on palms, about 3.5gm/square metre/day, than elsewhere (Goldschmidt & Kligman, 1963). A value for sole of feet was not determined.

1:5 Stratum corneum as a habitat (target tissue) for dermatophytes:-

Stratum corneum is the outermost layer of skin, its external aspect is in contact with the outside environment and its internal aspect with the inside (skin) environment. Skin is sterile at birth but soon becomes colonized by a

number of microorganisms which make up its flora. Dermatophytes are not considered part of this flora (Roberts, 1970; Midgley and Clayton, 1972).
The dermatophytes grow aerobically over a wide range of temperature (25-35°C) and pH (4-10) (Stockdale, 1955). Dermatophytes are not peculiar in their nutritional demand. Also they had been grown on sterile liver, muscle, kidney and spleen of guinea pigs (Jadassohn and Rehsteiner, 1931), hair (Vanbreuseghem, 1952; English, 1963), nail (English, 1963), dry powdered dermis and over the dermal side of excised human skin (Jacobs and Lorincz, 1957) in addition to excised skin (Blank, 1959) and stripped stratum corneum (Knight, 1973).
Dermatophytes were shown to have keratolytic (Weary and Canby, 1969; Yu, Ragot and Blank, 1972; Yu and Blank, 1973; Das and Banerjee, 1982;, Takiuchi et al., 1984; Asahi et al., 1985), other proteolytic (O'Sullivan and Mathison, 1971; Minocha et al, 1972; Skorepova and Hauk, 1986; Kunert and Kasafirek, 1988) and lipolytic activity (Nobre and Viegas, 1972; Das and Banerjee, 1977; Hellgren and Vincent, 1980). Serine proteinases (urokinase and tissue type plasminogen activator) which are involved in extracellular protein catabolism were found in dermatophytes and their release by the dermotophytes was suggested to play a major role in the invasion of the skin (Lotti et al, 1988). Sulphitolysis, a process that denatures keratin non enzymatically has been found during dermatophyte induced keratinolysis and suggested as a complementary mechanism to keratinolysis (Ruffin et al., 1976). Keratinase has been partially purified from and detected by immunoelectron microscopy in material from tinea pedis caused by T. rubrum (Koga et al., 1986). Using fluorescent antibody technique it was also detected in biopsies from skin of guinea pigs infected experimentally with T. mentagrophytes (Collins, Grappel and Blank, 1973). Disintegration of hair thought to be caused by dermatophytes enzyme digestion was reported in human scalp infection (Tosti et al 1970), experimental infection in guinea pigs (Poulain and Biguet, 1974) and in vitro (Baxter and Mann, 1969; Mercer and Verma, 1963; Verma, 1966). Thus it seems that dermatophytes have a battery of enzymes able to digest different materials in their habitat. Another character of dermatophytes is production of antibiotics which probably enable them to establish themselves in lesions (Youssef et al., 1979; Ryall et al., 1981; Hammedi, Howell, Noble, 1988) by eliminating other microbial nutritional competitors (Bibel and Smiljanic, 1979).
The dermatophytes in skin invade only stratum corneum. In skin generally there are a number of conditions some of which favour growth of dermatophytes while others do not.
Conditions favourable for growth of dermatophytes are:-

1. The stratum corneum is an avascular tissue composed of highly specialized, but dead cells. It is distant from the body's main defensive mechanisms.
2. It is well hydrated having water reaching it through eccrine sweating and transepidermal water loss. The temperature in it is cooler than the body (core) temperature of 37°C, pH ranges from 5.5 to 6.7 (Jolly, Hailey and Netick, 1961) and it is exposed to the aerobic condition of the atmosphere.
3. Stratum corneum is an agreeable tissue for growth of dermatophytes because it is composed of protein, amino acids, lipids, carbohydrate and various trace elements including iron (Monroe, et al., 1976).
4. Over some areas of 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 (Roberts and Mackenzie, 1987) for an airbourne dermatophyte infection (English, 1972). 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. This probably may account for the fact that distal subungal onychomycosis is the commonest clinical form of tinea unguium (Zaias, 1980) Thirdly, the interdigital spaces of toes, particularly the fourth and the crural area in male are naturally occluded and this may contribute to the fact that tinea pedis in most instances starts in toe webs (Kaaman, 1988) and tinea cruris is almost exclusively a male disease (Blank and Mann, 1975). Experimentally induced occlusion was found to cause the hydrated stratum corneum to swell and develop multiple folds (Harris, Papa and Stanton, 1974) and allowed accumulation of desquamated corneocytes on the surface of stratum corneum (Goldschmidt & Kligman, 1963; Montes and Wilborn, 1970). Therefore it is likely that occlusion increases the surface area and nutrients available for growth of dermatophytes on stratum corneum. Forthly in the pathological condition of palmoplantar hyperkeratosis which is characterized by a great increase in the thickness of the stratum corneum, there is a frequent occurrence of dermatophytosis (Nielsen, 1984).
5. There are certain cosmotic considerations which by altering the microenvironment of a delimited part of a stratum corneum may favour the growth of dermatophytes. Shoes, for example may act as an

artificial occlusion. Dermatophytosis may also commence under rings and wrist-watches (Berson and Grigoriu, 1976).

Conditions in skin which do not favour the growth of dermatophytes are:-

1. The epidermis is a proliferative tissue. The corneocytes are continuously shed from its surface. Therefore for dermatophytes to maintain their growth in lesions, they must at least keep pace with the rate of epidermopoiesis and exfoliation. Labelling indices showed enhanced rate of tritiated thymidine incorporation in the basal cells of the epidermis of the inflammatory margin of annular dermatophytosis in human (Berk, Penneys & Weinstein, 1976). In experimental bovine T. verrucosum infection increased rate of epidermal cell proliferation and desqumation of stratum corneum were reported (Lepper and Anger, 1976). In guinea pigs infected experimentally with dermatophytes, enhanced rates of epidermopoiesis were also found in lesion and correlated well with the severity of inflammatory changes; peak activity occurred at 10 days in the primary infection and at 2 days in reinfection and was subsequently followed by a spontaneous clearance after 10 day, (Tagami 1985). Furthermore in these experiments application of a heat-killed spores suspension produced inflammatory changes with enhanced epidermopoiesis only in immune animals and it was concluded that the dermatitic changes increased epidermopoiesis which facilitated the elimination of the fungus from the stratum corneum and that immune activity, particularly contact sensitivity to fungal antigens, exerted a crucial role in the induction of these changes. Poulain et al., (1980) reported the presence of mononuclear cells in the upper dermis after the healing of primary lesions in experimental dermatophytosis in guinea pigs, these were termed memory cells and were associated with the rapid clearance of secondary lesions. Recently, Hay, Calderon and Mackenzie (1988) found increased epidermal proliferation in experimental dermatophytosis in mice very early in the disease process and suggested that this pointed to a non immune mechanism and possibly amplified subsequently by cell mediated immunity.
   Normally the time required for the renewal of stratum corneum is about 2 weeks (Jansen, Hojyo-Tomoko & Kligman, 1974). A slow

rate of exfoliation may lead to the development of chronic infection by allowing the infection to establish itself before the corneocytes are shed (Rothman and Lorincz, 1963).

In ichthyosis, a group of diseases characterized by hyperkeratosis, dermatophytosis has been reported (Hay, 1982) and in psoriasis, a disease characterized by an accelerated epidermopoiesis, dermatophytosis was found to be of low incidence (Noble, 1981; Fransson, Storgards and Hammar, 1985). However a successful experimental inoculation of dermatophytes in psoriatic plaques in patients was obtained (Arieli, Alteras & Feurman, 1979) but the occlusion used may have had some inhibition on the epidermal turnover (Roberts & Mackenzie 1987). Other abnormalities are however, seen in psoriasis and these may affect dermatophytes growth e.g. tortuosity and dilation of the blood capillaries in the dermis with thinning of the suprapapillary plates may allow an increased diffusion of the reported serum antifungal substances into the stratum corneum and collections of polymorphonuclear leukocytes within or just below the stratum corneum may kill dermatophytes. The killing of germinated microconidia by phagocytosis and/or oxidative products of the respiratory burst of neutrophils and monocytes has been reported (Calderon & Hay 1987) and the neutrophils were found to be able to recognise, ingest and kill arthrospores (Richardson, personal communication).

2. Presence of Langerhans' cells in the skin makes it a possible site for the initiation of an immunological response (Sauders, 1983; Wolff and Stingl, 1983). Braathen and Kaaman (1983) reported that from patients with dermatophytosis, Langerhans' cells induced a proliferative T cell response to trichophyton stronger than that seen with macrophages. Holden, Hay and MacDonald (1981) detected antigenic substances along hyphal walls and also in tissue surrounding them in material from a dermatophyte infection and delayed-type hypersensitivity to keratinases of T. mentagrophytes was shown in guinea pigs previously infected with this fungus (Eleuterio et al., 1973). Emtestam., et al (1985) found an increase in the number of Langerhans' cells in the upper half of the epidermis along with their accumulation near dermatophyte elements in lesions of tinea cruris and suggested this as in agreement with the hypothesis that Langerhans' cells are responsible for the initial uptake and processing of antigen before

delivery to T lymphocytes in dermatophytosis. In a study analysing infiltrating cells in human dermatophytoses, Sugiura, Uehara and Watanabe (1987) found, in addition to the presence of dermal infiltrate of T helper and T suppressor cells, that in active margin of lesions Langerhans' cells were more in number in the upper dermis than in the epidermis and suggested this situation is similar to that seen in the active stage of allergic contact dermatitis where Langerhan's cells are known to migrate from the epidermis to the dermis. Thus it appears that in humans Langerhans' cells are involved in cell-mediated immune response against dermatophytes. The site of interaction between Langerhans' cells and T cells is not exactly defined, it could be the epidermis, dermis or regional lymph nodes.

3. Keratinocytes are known to carry out macrophage-like functions such as endocytosis and phagocytosis (Wolff and Honigsmann, 1971). They have been shown to phagocytose and kill Candida albicans (Csasto et al. 1986; Csasto, Kenderessy and Dobzy, 1987; Farkas Kemeny & Judak 1987). From the epidermis of guinea pigs a protein with fungicidal activity against C. albicans has been extracted (Chikakave et al, 1987). However such phagocytosis and killing of dermatophytes by keratinocytes has yet to be studied. Keratinocytes are also capable of releasing distinct immuno-modulating factors (cytokines) which have been suggested to play a role for initiation and maintenance of an early host defence reaction against, for example, microbial agents (Lauger et al; 1985), perhaps in association with Langerhans' cells in the postulated epidermal-Langerhans' cells unit (Wolff, 1972).

4. Sweat and/or its content of ascorbic acid (Cornbleet, Klein and Pace, 1936) has been shown to be inhibitory to growth of dermatophytes (Peck et al, 1939; Sanderson and Sloper, 1953a). Vitamin K was found to be inhibitory to growth of dermatophytes (Area-Leao and Furtado; 1950, Nekam and Polgar, 1950), and in sweat vitamin K-like substance was reported (Seutter and Sutorius, 1971).

5. Human sera had been reported to contain a factor inhibitory to growth of dermatophytes. This factor was found to be heat labile and dialysable (Lorincz, Priestley and Jacobs, 1958; Blank et al., 1959; Roth et al., 1959) and in skin explant it limited growth of dermatophytes to the stratum corneum (Blank et al., 1959). It was also found to be present in human sera regardless prior exposure

to dermatophytes (Carlisle et al., 1974). Serum transferrin was shown to be inhibitory to growth of dermatophytes from the different genera and to C. albicans and was suggested to work by binding iron which many organisms need for growth (King et al.,1975). Serum transferrin was however found to be heat stable and non dialysable unlike the previously reported serum factor. Yu, Grappel and Blank (1972) reported inhibition of keratinase of T. mentagrophytes by a2-macroglobulin which was present in sera from infected as well as non-infected adults and newborn babies. Thus it looks as if human serum may contain more than one dermatophyte inhibitory factor. Blank and Smith (1960) reported a patient deficient of serum antidermatophyte activity with a widespread T. rubrum infection. The rarity of scrotal involvement in dermatophytosis was attributed to diminished barrier function of scrotal skin allowing a more ready diffusion of serum antifungal factor to stratum corneum (Smith, Fischer and Blank, 1961). La Touche (1967), however, reported a common involvement of scrotum in tinea cruris and suggested that at this site cutaneous reaction is often inconspicuous and therefore requires careful examination. Nishimoto and Shinoda (1984) suggested the presence of a relatively small number of Langerhans cells in the scrotal skin as a contributory reason for the mild cutaneous reaction of the scrotum to a tinea lesion.

6. The skin surface is covered by an ultrafilm of lipids derived from sebaceous and epidermal origin. On palms and soles where there are no sebaceous glands dermatophytosis tends to be chronic.

   The spontaneous clearing of Microsporum audouinii scalp infection at puberty was ascribed to changes in sebum (Rothman and Lorincz, 1963). The role of sebum in the defence against dermatophytosis was not confirmed experimentally by Kligman (1963) who found that dermatophytes grew well in the presence of human sebum. Weary (1968) reported that Pityrosporum ovale, a normal member of the skin flora elaborated a substance that inhibited growth of dermatophytes in vitro. Roberts and Mackenzie (1987) suggested that the increased level of this lipophilic yeast on the scalp in teens may accord well with both Weary's report and the pubertal clearing and adult resistance to M. audouinii scalp infection. Clinically the inhibitory activity of fatty acids on dermatophytes is utilized in topical treatment of lesions by undecylenic acid and its derivatives (Lyddon, Gundersen and

Maibach, 1980; Chretien et al., 1980). Tinea capitis in India, unlike other clinical types of dermatophytosis, is less common and this was attributed to the use of some hair oil that was found to be inhibitory to the growth of dermatophytes in vitro (Hajini et al.,1970) and in experimental guinea pig infection (Abraham et al., 1975).

7. Carbon dioxide was found to be associated in vitro with checking of dermatophyte vegetative growth and formation of arthrospores (King et al., 1976). It diffuses physiologically from normal skin (Frame, Strauss and Maibach, 1972) and its level rises from damaged skin (Malten and Thiele, 1973). Under occlusion of skin, the level of carbon dioxide makes up 8-10% of the occluded atmosphere (Aly et al., 1978; King et al, 1978) and continuous occlusion was found to enhance clearance of experimentally induced dermatophytosis in guinea pigs (Kerbs and Allen, 1978). Also, prolonged occlusion was reported to induce changes in the skin pH and flora (Aly et al., 1978). The role of these changes in the clearance of dermatophytosis was, however, not studied.

1:6 Source of infection with dermatophyte fungi:-

Experimental human infection has been developed using different morphological forms of various dermatophytes over different body areas. Kligman, (1952; 1955) produced dermatophytosis on scalps using macroconidia and hyphal fragments of Microsporum canis grown in culture, naturally M. canis infected hair and spores "arthrospores" from naturally M. canis and M. audouinii infected hair as well as on back using naturally M. audouinii infected hair. Sloper (1955) induced lesions on forearms and legs by fragments of culture of Epidermophyton floccosum and on forearms by scales from T. mentagrophytes infection. Huppert and Keeney (1959) produced infections of T. mentagrophytes on the fourth interdigital spaces using pieces of culture of T. mentagrophytes. Desai and Bhat, (1961) used small fragments of mycelial growth of T. rubrum to induce infections on forearms. Ilowite (1967) produced lesions on soles using pieces of cultures of T. rubrum, E. floccosum and M. gypseum. Singh (1973) using both microconidia and mycelial fragments from a culture of T. rubrum produced lesions on forearms, outer wall of axilla and thigh. Using microconidia of T. mentagrophytes, lesions were produced on ankles

(Allen, et al., 1973), forearms (Jones et al., 1974 a, 1974 b, and 1974 c; Reinhardt et al., 1974; Bibel and LeBurn, 1975) and lumbar areas (Reinhardt et al., 1974). Reinhardt et al., (1974) also used T. mentagrophytes infected scales to produce lesions on ankles, thighs and forearms. Thus it can be concluded that the different morphological forms of dermatophytes have the potential of causing human infection but arthrospores, because of their in vivo formation and shedding from lesions, are likely to be the forms involved in the spread of the infection. In lesions the dermatophytes exist also as hyphal elements and these too are shed and since a direct contact with a lesion is considered as one mode by which dermatophytoses spread it seems probable to assume a role for hyphae in this mode of spread of dermatophytosis. In the indirect mode of spread of dermatophytosis i.e. contact with shed infected material, the role of arthrospores is substantiated by the fact that beside being spores, i.e. non-vegetative thus having no nutritional requirements, they are resistant to adverse conditions (Hashimoto and Blumenthal, 1978). They can also be produced in large numbers especially when hair is involved where myriads of them have been observed (Shelly, Shelly and Burmeister, 1987). Austwick (1963) calculated that some 30,000 arthrospores could be present per millimetre length of infected hair in cattle ringworm. 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. Fungistasis (soilstasis), antibiosis, competition and lysis are factors known to govern microbial survival in the environment (Griffin, 1972). Grin and Ozegovic (1963) found among dermatophytes pathogenic to human and animals only M. gypseum and T .mentagrophytes survived in unsterilized soil. On the other hand, in laboratories various dermatophytes remained viable for long periods in infected skin scales and hair stored under dry, dark conditions (Rosenthal and Vanbreuseghem, 1962; Dvorak, Hubalek and Otcenasek, 1968). Whether sexual (perfect) stages of dermatophytes have a role in the causation of dermatophytosis has not been explored. They have been studied in the laboratory where two compatible strains are grown together and so far none have been found for the anthropophilic dermatophytes. Of the pathogenic dermatophytes M. gypseum has two sexual stages Nannizia (Arthroderma) gypsea and Nannizia (Arthroderma) incurvata and is known to be present in the soil widely (Ajello, 1956, 1960;). On one occasion cleisothecia of M. gypseum (Nannizia incurvata) were observed in a soil sample with added horse mane hair after 8-10 days incubation at 24°C (Gentles, 1962). This sample was collected from a soil suspected of being the source of an outbreak of M. gypseum infection (Alsop and Prior, 1961). Since

the dermatophytes are heterothallic it appeared that compatible strains were present in that soil suggesting the possibility of cleistothecial (ascospores and peridial hyphae) formation in it naturally. Moreover the experimental conditions under which they were observed are environmentally not uncommon. This possibility therefore argues a role for sexual (perfect) forms (ascospores and/or peridial hyphae) of M. gypseum in the initiation of at least that outbreak. However it is thought that the encounter of sexually compatible strains in nature in circumstances favourable to the production of the sexual state occur only rarely (Nicot, 1981). Alternatively, other vegetative saprophytic forms may be the means in which M. gyseum is acquired from soil. Gordon, Ajello and Georg (1952) recovered from a soil several macroconidia typical in all respects to those of M. gypseum and isolated this dermatophyte in culture from the same soil. Otherwise, M. gypseum infection is acquired by exposure to the parasitic forms of the fungus, i.e. infected materials shed into a soil from human or particularly animals lesions. It is a common cause of infection in animals, dogs, cats, horses and other lower animals, (George, 1960; Kaplan, 1967) and chances of its isolation from soil samples increased with the presence of animals at the collecting site (Ajello, 1953). Man to man contact, i.e. via parasitic forms of the fungus, also was believed to be the route by which M. gypseum infection subsequently spread in outbreaks (Whittle, 1953; Alsop and Prior, 1961). A patient who was confined to bed without having the chance of being in contact with soil has developed M. gypseum infection of the scrotum (Nishimoto and Shinoda, 1984).

1:7 Spread of dermatophytosis:-

Many species of dermatophytes have been identified but only a few cause human infection and these fall "broadly" into three epidemiological groups, geophilic, zoophilic and anthropophilic. The geophilic species are present in the soil and along with other keratinophilic soil fungi decompose keratinous debris. Of the geophilic dermatophytes M. gypseum is the most commonly involved in human dermatophytosis but it remains a rare cause in comparison to the dermatophytes of the other two groups. Small outbreaks of infection however have been reported (Whittle, 1954; Alsop and Prior, 1961; Sierra De Arroyave et al., 1977).

The zoophilic species are commonly encountered as animal pathogens but infection can be passed directly or indirectly to humans. Human to human

transmission of zoophilic dermatophytes occurs (Kaplan, 1967) but is thought to have a limited epidemiological significance (English, 1972). . Perhaps this is because of the shorter courses of zoophilic infection in humans thereby allowing a comparatively short time for the occurrence of this type of transmission.

The anthropophilic species are primarily human pathogens spreading by direct contact or indirectly through sharing of infected fomites or exposure to contaminated surroundings. Infection of animals by anthropophilic dermatophytes is not common but has been reported and in some instances traced directly to human sources (Vogel and Timpe, 1957; Kaplan, 1967; Kushida and Watanabe, 1975; Thakur and Verma, 1984;Stenwig and Taksdal, 1984; Ogbonna, Enweani and Ogueri, 1986; Nooruddin and Singh, 1987) and in others transmitted to humans (Nonma, Nishimoto and Imafuku, 1987). Perhaps the inaccessibility of human lesions and materials shed from them to animals are factors responsible for the uncommon occurrence of anthropophilic infection in animals because experimental infections with T. rubrum, T. tonsurans and E. floccosum have been consistently produced in guinea pigs (Chittasobhon, and Smith, 1979) and in dogs (T.rubrum) (Kushida and Watanabe, 1975). Dawson, (1968) in a discussion of ringworm in animals cited instances of occurrence of anthropophilic dermatophytes (T. rubrum, M. auduoinii, T. violaceum, T. schoenleinii, E. floccosum) in farm, laboratory and wild animals and believed that the demarcation between the anthropophilic and zoophilic species is not absolute.

Shedding of dermatophytes from lesions was shown by Rosenthal et al., (1956) in washings from feet of infected persons and their occurrence in places and fomites suspected of being involved in the transmission of infection has been reported. A springboard in one survey yielded the highest degree of dermatophytes isolated from a swimming pool (Detandt and Nolard,1988). Tinea pedis in a husband was transmitted as tinea corporis of the buttock to his wife because the husband used to treat his foot while placing it on the toilet seat (Rothman, Knox and Windhorst, 1957). Patients with tinea pedis deposit dermatophytes in their socks and shoes which then form a reservoir of infection (Ajello and Getz 1954; Broughton, 1955; Knudsen, 1986) and infected cattle deposit hair and scales containing T. verrucosum in scratching posts which is then believed to become a source for the indirect spread of this type of infection (Walker, 1955). Dermatophytes have been recovered from the floors of communal bathing places (Gentles, 1956 and 1957; Detandt and Nolard, 1988). In a survey of T. tonsurans scalp infection in a residential school, Mackenzie (1961) isolated

this dematophyte not only from hairbrushes, combs, bed clothes, but also from the floor and a curtain, and believed that bedmaking and air currents could have been responsible for the distribution of the infection. Dispersal of dermatophytes into the air has been shown by their recovery from the atmosphere of dermatology clinics where skin scrapings were taken (Friedman et al., 1960; Midgley and Clayton, 1972) and in a pet shop which had housed infected animals (Uscavage and Karl, 1961). Dermatophytes have also been isolated from house dust of patients infected with them (Shirouchi and Murata, 1987). In animal houses, too, dissemination of dermatophytes from experimentally induced (Chittasobhn and Smith, 1977) and naturally occurring (Dawson and Noddle, 1968) lesions in animals into the air and onto non contact animals occurred and airbourne infection resulted (Rebell et al., 1956).

1:8 Development of dermatophytosis:-

1:8:1 Site of infection:-

Dermatophytes grow in non-living cornified tissue, stratum corneum of skin, hair and nails in the form of hyphae forming arthrospores.

In the skin they remain confined to the stratum corneum. In nodular granulomatous perifolliculitis of legs presence of a dermatophyte (T. rubrum) in the dermis is due to rupture of follicular walls and extrusion of the dermatophyte into the dermis with a subsequent granulomatous reaction (Wilson, Plunkett & Gregersen, 1954). On the basis of the histopathological similarity of nodular granulomatous perifolliculitis of legs to Majocchi's granuloma Wilson et al. (1954) regarded the former as a variant of the latter which occurs elsewhere in the skin. A similar mechanism was ascribed to the very rare condition of the so-called dermatophyte mycetoma (pseudomycetoma), (Ajello, Kaplan and Chandler, 1980). In the suppurative infection caused by Trichophyton verrucosum, the dermatophyte remain confined to the hair follicles (Birt and Wilt, 1954).

Hadida and Schousboe, (1959) described under a name of maladie dermatophytique, deep chronic dermatophyte infections of subcutaneous tissue, lymph nodes, internal organs and bones with a fatal outcome. A few more cases of deep involvement have been reported (Araviysky, Araviysky and Eschkor, 1975; Swart and Smit 1979; Hironaga et al., 1983), and in those patients who have been investigated thoroughly defective cellular

immunity or deficiency of serum transferrin was found. In these cases of deep involvement dermatophytes grew as branching hyphae and this shows the ability of dermatophytes to behave like agents of systemic mycoses in an invasive filamentous form. Therefore in patients of debilitating illness dermatophyte infection especially if chronic should be taken seriously and treated effectively.

Diabetes mellitus is thought generally to be associated with an increased incidence of dermatophytosis. Vascular insufficiency is a common feature in diabetics and was suggested to be an important factor in the development of onychomycosis (Daroczy and Herpay, 1978). In a study of prevalence of dermatophytosis in toewebs and toenails of diabetic and diabetes free patients there was, however, no significant difference between the two groups (Alteras and Saryt, 1979). Perhaps the importance of dermatophytosis in diabetics is the destruction of the structural and functional organization of stratum corneum as a protective barrier therefore providing a portal of entry for other pathogenic organisms which may cause for example gangrene or other infections.

It is not uncommon for two or more distant sites to be involved in the same patient, e.g. tinea cruris and tinea pedis in T. rubrum infection (Rosman; 1966), tinea capitis and tinea corporis in T. tonsurans infection (Bronson, et al., 1983), and one hand, two feet presentations of T. rubrum infection (Chapel and Chapel, 1985; Goslen and Kobayashi, 1987). In such cases one site might have served as a source of the inoculum which may have infected the second site, i.e. autoinfection.

Dermatophytosis develops also in clinically unsuspected sites, e.g. diaper area of babies (Cavanaugh & Greeson, 1982; Parry, Foshee & Marks, 1982), eyelids and eyelashes (Montgomery, 1945), upper lip (Kubo et al., 1984), pinna (Verbov, 1973), external auditory meatus (Watanabe, 1986) and glans penis (Dekio and Jidoi, 1989).

Infection with two distinct dermatophytes has been reported in the same (Crozier and Searls, 1979; Kubo et al., 1984; Tschen, Head and Macdonald, 1986; Saul, Bonifaz and Arias, 1987) or different sites (Crozier and Searls, 1979) and so mixed dermatophyte and C. albicans infections (Crozier and Searls, 1979; Saul et al., 1987). In patients with chronic mucocutaneous candidosis, dermatophytosis of various body sites occur too (Shama & Kirkpatrick, 1980).

T.rubrum, T. interdigitale, E. floccosum and to a certain extent M. canis are the dermatophytes commonly involved in infection of a chronic nature (Hay, 1982a; Kaaman, 1988). Young and Roth (1979) have found immunological cross-reactivity between human blood group active

glycoprotein (isoantigen A) and glycoprotein isolated from cell walls of T. mentagrophytes, T.rubrum and E. floccosum. They suggested that in those subjects who possess tissue antigens which cross react with the fungus immunological tolerance may allow the dermatophyte to proliferate unchecked and recommended the inclusion of blood typing in studies of chronic fungal infections.

Recently, progesterone has been reported to inhibit growth of dermatophytes in vitro and this inhibitory action was suggested to be operative in vivo because of the increased incidence of dermatophytosis in females after menopause (Stevens, 1989).

1:8:2 Hair invasion:-

Two types of hair invasion are recognized, ectothrix and endothrix and in both, dermatophytes do not penetrate below the zone of keratinization. In the ectothrix type, arthrospores are produced around the hair and it is clear from Kligman's (1952 ; 1955) work on M.auduoinii and M. canis scalp infection (ectothrix) hair is invaded from the adjacent stratum corneum. In the endothrix type, arthrospores are produced inside the hair but from where the hair is invaded has not been studied. However, it has been pointed out that there could not be endothrix invasion of hair without hyphae being at first developed ectothrixically (Kolemen, personal communication). In tinea faciei in adult males (tinea barbae) coarse hair of the beard and moustache area is involved in a manner thought to be similar to that of tinea capitis (Roberts and Mackenzie, 1987). Terminal hair can be involved in tinea corporis (Roberts and Mackenzie, 1987) and infection of vellus hair has also been reported (Eby and Jetton, 1972).

1:8:3 Nail invasion:-

Nails in distal and proximal subungual onychomycosis are invaded from the adjacent stratum corneum (Zaias, 1972). Formation of tunnels and spaces in infected nails has been observed (Alkiewicz and Swarinski, 1967). In white superficial onychomycosis, the dermatophyte, T. mentagrophytes commonly, invades the superficial surface of the nail plate, a toe nail mostly, resulting in the formation of powdery materials that can be scraped away and it grows in forms (hyphal fronds) that resemble those of its growth in nail in vitro (Zaias, 1972 ; 1980; English 1963). Circulatory disturbances in the extremities were suggested as an important contributory factor in the

susceptibility to and resistant to treatment in onychomycosis (Daroczy and Herpay, 1978). Nail infections because of their chronicity may serve as a reservoir for a geographically restricted dermatophytes in non endemic areas. Kalter and Hay (1988), for example, reported onychomycosis due to T. soudanense in patients of African origin after years of living in England.

1:9 Diagnosis of dermatophytosis:-

Dermatophytosis clinically mimics other different dermatological diseases. Suspected lesions therefore should be examined for dermatophytes before administering specific therapy. Under Wood's light, hair infected with M. auduoinii or M. canis emits brilliant green fluorescence and with T. schoenleinii pale green fluoresence. Wood's light is also a useful tool in investigating an outbreak of tinea capitis, for example in school children.
Examination of a lesion for the presence of a dermatophyte can basically be considered as a three step procedure. At first microscopical examination of material from the lesion is carried out, then material is cultured and lastly identification of the isolated dermatophyte. Biopsies are rarely needed for a diagnosis of dermatophytosis.
Materials (scraped skin scale, plucked and broken hair, clipped and scraped nail fragments) gathered from a lesion by a scalpel and forceps are mounted in potassium hydroxide (10-30%) to clear the corneocytes and examined microscopically for the presence of hyphae and arthrospores. It is preferred to obtain material from the margin of a lesion especially if it is expanding. Culture is done by inoculating fragments of the gathered material on multiple areas on the growth medium which commonly is glucose peptone agar (Sabouraud's dextrose agar) or malt extract agar. Cultures are incubated at 28°C and kept for at least three weeks before discarded as negative. Identification of the isolated dermatophyte depends on its colonial appearance and more importantly on its micromorphology. By mounting small fragments of a cultured dermatophyte colony in lactophenol-cotton blue, after a drop of alcohol is added initially to prevent the formation of air bubbles, the arrangement of conidia and presence of accessory structures are examined microscopically and the dermatophyte is identified accordingly as discussed in section 1. Modifications of this three step procedure have been reported. Vinyl adhesive tape (Knudsen, 1975), cyanacrylate contact cement (Whiting and Biset, 1974) have been used to collect material from lesions and similarly moistened gauze was used to obtain hair from scalp lesion

(Borchers, 1985). Likewise various stains e.g. chlorazol Black E (Burke and Jones, 1984) and the fluorescent whitening agent, Blankophor (Gip and Abelin, 1986) have been used with potassium hydroxide to mount material for light and fluorescent microscopy and cultures of dermatophytes were obtained from vinyl adhesive tapes employed in sampling lesions (Knudsen, 1975). Overall the results obtained in terms of dermatophyte isolation from suspected materials by these methods are not superior to those of the conventional three step procedure.

1:10 Treatment of dermatophytosis:-

Localized lesions of dermatophytosis are treated with topical antifungal preparations of which there is a wide variety ranging from the traditional Castellani's paint, Whitfield's ointment and undecylenic acid to the modern azole derivatives for example clotrimazole, econazole and miconazole. Widespread and intractable scalp and nail infections are generally treated with griseofulvin in a dose of 10mg/kg/day for children and 0.5-1gm for adults daily. Theoretically treatment should continue till the infected stratum corneum, hair and nail outgrow the infection. This is taken generally as a minimum of four weeks for body lesions, six weeks for scalp infection and 12 months for nail infection or even longer in case of toe nails. Adding topical treatment may enhance recovery as may clipping away and manual epilation of infected scalp hair. Recently the use of concentrated urea (40%) as a mean of atraumatic avulsion of infected nail along with bifonazole under occlusion has been reported for the treatment of onychomycosis with encouraging results (Nolting, Stettendorf and Ritter, 1986; Roberts et al; 1988).

Ketoconazole is an alternative for griseofulvin in the systemic treatment of widespread and intractable dermatophytosis but it has a serious side-effect of hepatic damage.Development in the treatment of fungal diseases is continuing. The allylamines, for example, terbinafine is a recent addition to the therapy of dermatophytosis (Villars and Jones, 1988).

1:11 Aim of the study

In lesions the dermatophytes grow in the stratum corneum and form arthrospores. Little is known about the events involved in the colonization and invasion of stratum corneum by dermatophytes. Because of their in vivo formation and shedding from lesions, arthrospores are thought to play a major role in the spread of dermatophytosis. Therefore they are preferred to be utilized in studies concerning dermatophytosis and its treatment.

From lesions of dermatophytosis, corneocytes and hair are shed along with arthrospores and a role for substances in corneocytes (Hashimoto and Blumenthal, 1978) and in hair (Dvorak, Hubalek and Otcenasek, 1968) in protecting the arthrospores shed with them have been suggested but not investigated.

Dormancy is a property ascribed to spores in general whereby under conditions unsuitable for germination but not lethal they retain their ability to germinate once these conditions become suitable to do so. Dormancy of arthrospores under conditions comparable to the external environment into which they are shed from lesions has not been investigated.

Hydration is an essential element for the germination of fungal spores. Arthrospores shed from lesions into wet places e.g. floors of swimming pools and communal bathing facilities and into damp items, e.g. used towels are subjected to moist conditions. A role for the initial hydrated state of arthrospores in the spread of dermatophytosis in places or by items such as those mentioned above has not been studied.

Laboratory animals and media are commonly employed in studying antidermatophyte activity of drugs. The utilization of human excised skin and stripped sheets of stratum corneum as media to test for antidermatophyte activity of drugs has been reported (Kligman 1986; Knight, 1973 ; 1974). The reported results encourage further development in this fully unexplored field.

The aims of the study can be summarized as follows:-

1. To study methods of arthrospore formation in vitro. This study may help to understand the biological aspects of arthrospore formation in vivo.
2. To study the events involved in the colonization and invasion of the stratum corneum by dermatophytes; investigating, firstly, adherence of arthrospores to corneocytes; secondly, germination of arthrospores in suspensions of corneocytes and finally, growth of arthrospores in

stripped sheets of stratum corneum. The ultrastructure of the corneocyte-dermatophyte relationship was determined using scanning and transmission electron microscopy. Corneocytes from the body areas which are used in the clinical classification of dermatophytosis were employed.

3. To study whether corneocytes offer a protective coat to the arthrospores shed with them from lesions by testing the survival of arthrospores under certain adverse conditions in the presence and absence of human corneocytes.
4. To investigate the effect of an exogenously imposed dormancy on the subsequent germination of arthrospores. This along with item 3 may cast light on longevity and infectivity of arthrospores shed into the environment.
5. To study the effect of an initial hydrated state on the germination of arthrospores. This may further contribute to the present understanding of the increased incidence of dermatophytosis of feet in people sharing common recreational and bathing facilities.
6. To explore the possibility of developing further for testing of anti-dermatophytes drugs, a model which uses the target tissue of dermatophytes i.e. stratum corneum, as the growth medium.

Chapter 2

Production and Development of Arthrospores

2:1 Summary:-

Arthrospore formation in 11 strains of Trichophyton interdigitale and six strains of Trichophyton mentagrophytes was tested using a number of growth conditions atypical for culturing dermatophytes. The conditions tested were incubation at 37°C, elimination of glucose from growth media, presence of amphotericin-B in growth media and increased carbon dioxide tension (10%) in the incubation atmosphere. With the exception of amphotericin-B all the tested conditions were associated with arthrospore formation. The results are discussed with remarks to the presence in the skin of factors which resemble the experimental conditions. A new procedure of incubation of dermatophytes under an atmosphere enriched with carbon dioxide is described.
An ultrastructural study of arthrospores is also presented.

2:2 Introduction:-

Fungi consist of vegetative and reproductive structures. Vegetative structures may be filamentous, as in dermatophytes or unicellular as in yeasts. Reproductive structures are spores which can be produced sexually or asexually. Spores can be carried by air, water, insects or animals even for long distances so they are also agents of fungal dispersal. Certain fungal spores additionally serve as infectious particles for man in which cutaneous, subcutaneous or systemic fungal diseases may develop. Fungal spores are of different sizes and shapes and they may be produced in large numbers. More than one type of spore may be produced by the same fungus. Dermatophytes, for example, produce microconidia, macroconidia, chlamydospores, and arthrospores asexually and ascospores sexually. They are all of different sizes and shape. While micro and macroconidial and chlamydospore formation is seen usually under cultural conditions, arthrospore formation is not but rather is characteristic of dermatophyte infection of skin, hair or nails. Ascospore formation on the other hand occurs when two compatible strains designated as + and - (A and a) come in contact under conditions hostile for vegetative growth, e.g. unsterilized soil, and so far observed only in laboratory culture.
The mechanisms involved in formation and liberation of spores from their parent thalli are different. Principally, there are, however, two types: blastic

and thallic development (Cole, 1986). Blastic development occurs as described by Cole, (1986) apically or laterally by blowing out and de novo growth of part of a fertile hyphae which becomes later delimited by a basal septum from the parent hypha. Blastic conidia secede from their parent hyphae by centripetal splitting of this basal septum, a process referred to as schizolysis. Thallic development occurs by either conversion of an entire segment into a single, terminal or intercalary conidium or into several conidia. The former is described as a holothallic conidium and the latter as an arthric conidium. Thallic conidia secede by either schizolysis or rhexolysis (rupture of a cell adjacent to the developed conidium). In arthric conidia the original hyphal wall either remains as part of the conidial wall or detaches eventually from the newly endogenously-formed conidial wall. These two modes of the thallic-arthric development are respectively called holoarthric and enteroarthric. Micro and macroconidia of dermatophytes were variously described, as holothallic by Cole and Samson (1983), and holoblastic by Galgoczy (1975; 1978) who also excepted lateral macroconidia of Epidermophyton floccosum and placed them in an intermediate position between holothallic and holoblastic. The situation of chlamydospores of dermatophytes with regard to the mode of their formation and liberations is so far not established. Development of arthrospores in dermatophytes (T. mentagrophytes) has been studied by Bibel et al., (1977) and Hashimoto et al., (1984). The latter group found that the original hyphal wall ultimately became sloughed from the newly formed arthrospore wall and concluded that arthrospore ontogeny in this fungus is enteroarthric and not holothallic as previously thought.

The initial sign of arthrosporogenesis as has been described by Hashimoto et al, (1984) was a deposition of what they termed a conidium specific wall layer on the inner surface of a pre-existing hyphal wall. The invaginating septal material was found to be continuous with the newly deposited layer but not with the outer hyphal wall. Separation of arthrospores occurred at their septa (schizolysis).

Under normal cultural conditions dermatophytes usually do not form arthrospores. However, in vitro formation of arthrospores had been reported under conditions unusual for culturing dermatophytes (Bibel et al., 1977, Emyanitoff and Hashimoto, 1978; Weigl and Hajtmanek, 1979 ; 1980; Wright, Scott and Gorman, 1984; Fujita Matsujama and Sato, 1986). These unusual cultural conditions were: incubation temperature of 37°C, elimination of glucose from growth media, presence of sublethal doses of antifungal drugs in growth media, and an increased carbon dioxide (CO_2) tension in the incubation atmosphere. In this study these conditions were

tested for arthrospore production in strains of T. interdigitale and T. mentagrophytes

2:3 Aim of the study:-

The aim of this study was to find out a method which would provide an abundant formation of arthrospores in strains of T. interdigitale and T. mentagrophytes and to adopt it as the standard procedure of production of arthrospores. Because the ultrastructure of arthrospores is not particularly well established this was studied by scanning and transmission electronmicroscopy.

2:4 Materials and methods:-

2:4:1 Strains

Eleven strains of Trichophyton interdigitale and six strains of Trichophyton mentagrophytes were used in this study. The strains of T. interdigitale were fresh isolates from clinical specimens (skin scale and nail clippings of feet and toe nails lesions) sent to the Medical Mycology Unit, Department of Dermatology, University of Glasgow, U.K. In this study they were numbered 1 to 11 respectively. The six strains of T. mentagrophytes were kept in silica gel at 4°C. Their stock numbers, 105, 115, 116, 118, 121 and 126, were maintained.

2:4:2 Growth media:-

The growth media used to study arthrospore formation at 37°C and in the presence of amphotericin-B were Sabouraud's dextrose agar (SDA) and broth (SDB) (Appendix). Amphotericin-B, 25mg, was initially dissolved in 10ml dimethyl sulphoxide and then 1ml added to 500ml of growth medium after it has been cooled. The medium used to study arthrospore formation in the absence of glucose was 4% peptone broth (Appendix) and under an increased carbon dioxide (CO_2) tension in the incubation atmosphere was SDA.

2:4:3 Inoculation of media:-

Initially, the six strains of T. mentagrophytes were recovered on SDA at 28°C from silica gel. Subcultures were made on SDA for all the strains and incubated at 28°C for two weeks to obtain a considerable yield of microconidia. Colonies were scraped off with a sterile surgical blade and dispersed into sterile distilled water. Each suspension was mixed vigorously for a few minutes to disperse clumps. One ml of this suspension was either plated evenly on SDA or pipetted into broth. For the strains of T. interdigitale, all the four growth conditions were used and for the strains of T. mentagrophytes CO_2 was used only.

2:4:4 Incubation of media:-

With the exception of media which were to be incubated at 37°C, incubation was carried out at 28°C. At 37°C, additionally SDB inoculated with strain number 2, 3 and 4 was incubated on a gyratory shaker (100 rev/min). Incubation under increased CO_2 tension was carried out in a modular incubator chamber (Figure 2:1) gassed with 10% $C0_2$ in air from a cylinder. Gassing of the chamber was undertaken on a daily basis. For all the four cultural conditions incubation was carried out for 10 days. To examine whether arthrospore formation would occur if the strains were grown under ordinary cultural conditions control media, SDA and SDB for T. interdigitale strains and SDA for T. mentagrophtyes, inoculated in the manner described above were incubated aerobically at 28°C for 10 days.

2:4:5 Electronmicroscopy of arthrospores:-

Samples from a colony of T. mentagrophytes strain number 121 cultivated under 10% CO_2 in air for 8 days were examined under transmission and scanning electron microscopy using standard methods of preparation.

2:4:6 Assessment of results:-

After preliminary experiments, arthrospore formation under CO_2 in some of the strains was found to be very abundant, with almost complete disarticulation of the entire culture into arthrospores. Therefore attempts to record the results by identifying hyphal tips and determining the number breaking down into arthrospores was not possible. Instead the results were

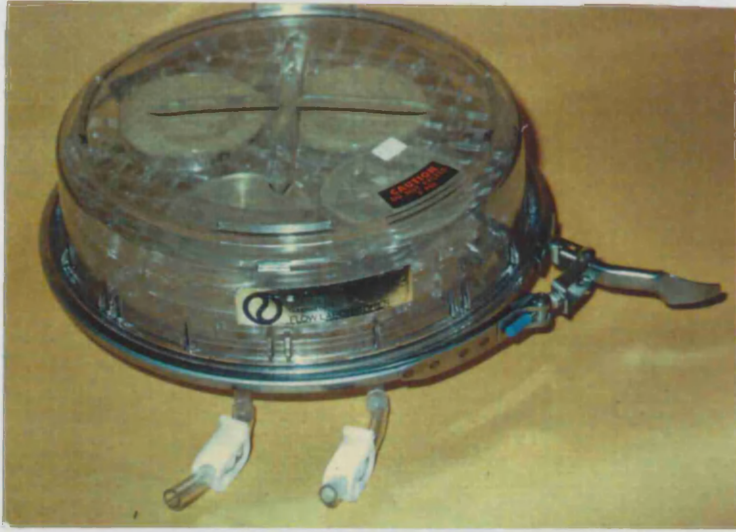


Figure 2:1:- Modular incubator chamber

recorded, based on making Lactophenol-cotton blue mounts from three different locations of a culture on agar or broth, as follows:

	= no arthrospore formation
+	= low degree of arthrospore formation
++	= mild degree of arthrospore formation
++++++	= very abundant arthrospore formation

2:5 Results:-

2:5:1 Effect of temperature (37°C) on arthrospore formation:-

Three strains (2, 3 and 4) of T. interdigitale produced arthrospores to a mild degree (Figure 2:2, Table 2:1) at 37°C on SDA and SDB. In SDB for all the strains, many round structures, "chlamydospores" were formed (Figure 2:3). They were larger than arthrospores and especially numerous in the agitated SDB for strain 2, 3 and 4. Agitated incubation was done only for strain 2, 3 and 4 because they were the only strains which formed arthrospores at 37°C and it was questioned whether or not it could be associated with formation of more arthrospores. Its inclusion in the remainder of the strains was abandoned because of the likelihood of the formation of these round structures and the difficulty which would be encountered in the separation of arthrospores. Furthermore the arthrospores remained in chains requiring methods to disarticulate them actively, e.g. by glass bead-vortemixing, which could have damaged and therefore rendering them unsuitable for the proceeding experiments of this project.

2:5:2 Effect of amphotericin-B on arthrospore formation:-

Arthrospore formation was not observed in any of the strains of T. interdigitale in the presence of amphotericin-B in growth medium (SDA and SDB).

2:5:3 Effect of glucose elimination on arthrospore formation:-

Only the three strains (2, 3 and 4) which formed arthrospores at 37°C did so in media lacking glucose (4% peptone broth). The degree of arthrospore formation was low (Table 2:1).

Species	Strain	37°C			↓Glucose	Amphotericin-B		10% CO_2	Control	
		Agar	Broth	Agitated	Broth	Agar	Broth	Agar	Broth	Agar
Trichophyton interdigitale	1	-	-	ND	-	-	-	++	-	-
	2	++	++	++	+	-	-	++++++	-	-
	3	++	++	++	+	-	-	++++++	-	-
	4	++	++	++	+	-	-	++++++	-	-
	5	-	-	ND	-	-	-	-	-	-
	6	-	-	ND	-	-	-	++	-	-
	7	-	-	ND	-	-	-	-	-	-
	8	-	-	ND	-	-	-	-	-	-
	9	-	-	ND	-	-	-	-	-	-
	10	-	-	ND	-	-	-	-	-	-
	11	-	-	ND	-	-	-	-	-	-
T. mentagrophytes	105	ND	ND	ND	ND	ND	ND	++	-	-
	115	ND	ND	ND	ND	ND	ND	++	-	-
	116	ND	ND	ND	ND	ND	ND	++	-	-
	118	ND	ND	ND	ND	ND	ND	++	-	-
	121	ND	ND	ND	ND	ND	ND	++++++	-	-
	126	ND	ND	ND	ND	ND	ND	++++++	-	-

Table 2:1: - Arthrospore formation under various cultural conditions.

– = No arthrospores

\+ = Formation of few arthrospores

++ = Formation of arthrospores in a mild degree

++++++ = Very abundant formation of arthrospores

ND = Not done

↓glucose = Glucose was eliminated from media

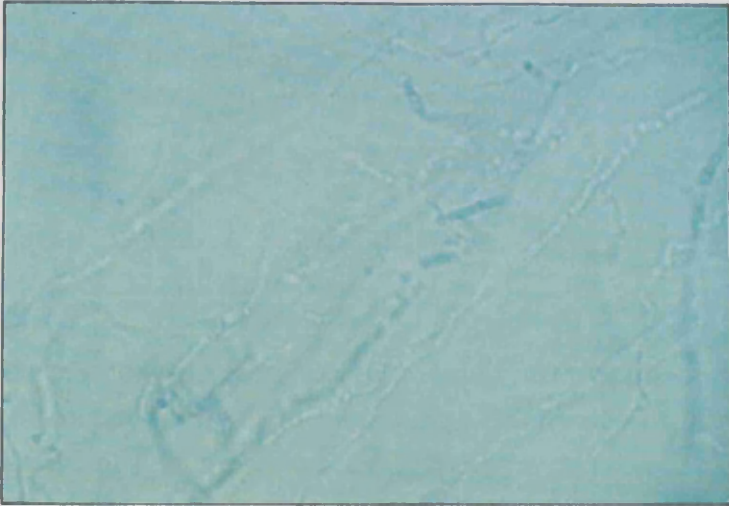


Figure 2:2:- Formation of arthrospores of T. interdigitale strain 4 on SDA after 10 days incubation at 37°C. Lactophenol cotton-blue mount, X 252.

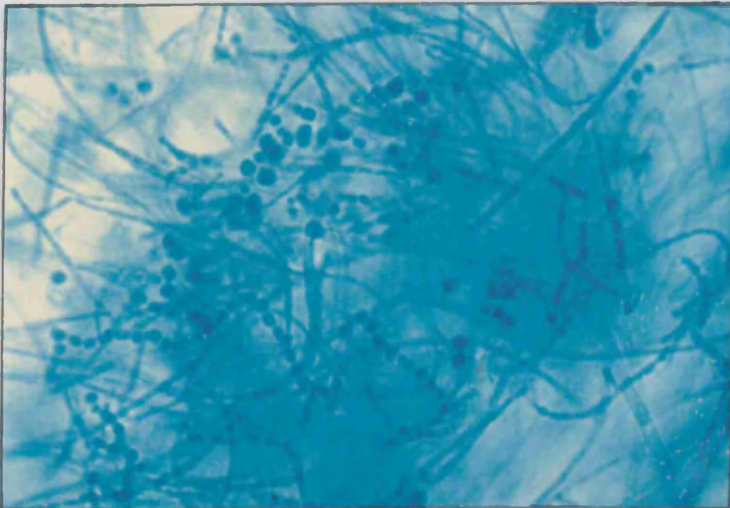


Figure 2:3:- Formation of arthrospores and chlamydospores of T. interdigitale strain 4 in SDB after 10 days incubation at 37°C on a gyratory shaker. Lactophenol cotton-blue mount, X 252.

2:5:4 Effect of 10% CO_2 on arthrospore formation:-

Five of the T. interdigitale strains (1, 2, 3, 4, & 6) produced arthrospores under 10% CO_2, 1 and 6 mildly, 2, 3 and 4 very abundantly (Figure 2:4; Table 2:1). All the strains of T. mentagrophytes produced arthrospores under 10% CO_2, 121 and 126 very abundantly and the rest mildly (Table 2:1).

In those strains where arthrospores formation was graded as very abundant the colonies were composed of almost completely disarticulated arthrospores.

2:5:5 Micro and macroconidial formation under various cultural conditions:-

Apart from those strains which formed arthrospores very abundantly, micro and macroconidial formation was observed in the remainder in SDA including those which formed arthrospores mildly.

2:5:6 Hyphal growth under various cultural conditions:-

Apart from those colonies which were composed of almost completely disarticulated arthrospores, aseptate hyphae were seen in the rest including those in which arthrospore formation was mild.

2:5:7 Control media:-

Controlled media showed the normal morphology of Trichophyton species.

2:5:8 Ultrastructure of arthrospores:-

Under the scanning electronmicroscope arthrospores appeared mainly round and encircled by indentations (Figure 2:5). Disarticulated arthrospores were seen connected by fibrils (Figure 2:6).

By transmission electronmicroscopy arthrospores were seen surrounded by a thick wall. Clear plasma membranes were also seen (Figure 2:7).

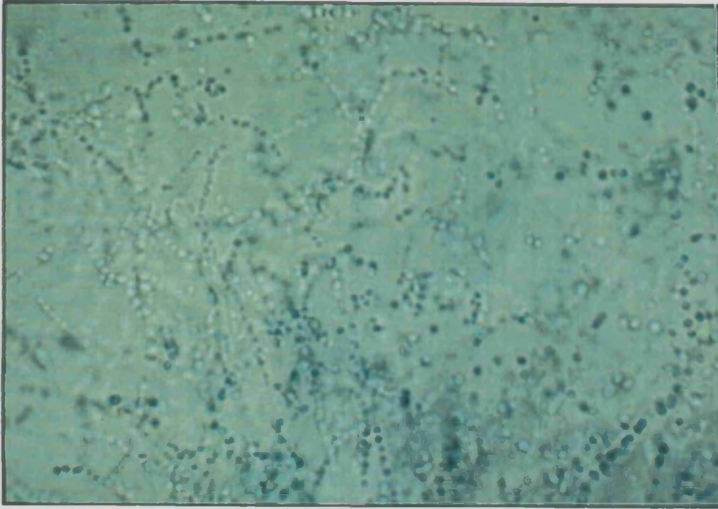


Figure 2:4:- Prolific formation of disarticulated arthrospores of T.mentagrophytes strain 121 on SDA after 10 days incubation under an increased CO_2 tension (10%). Lactophenol cotton-blue mount, X252.

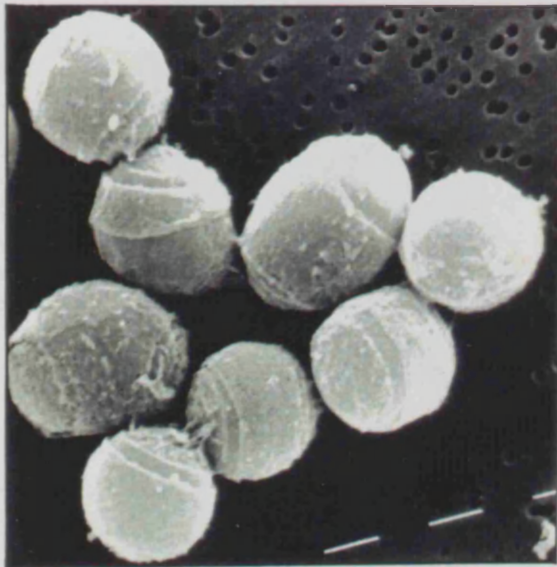


Figure 2:5:- Scanning electronmicrograph of arthrospores of T.menta-grophytes strain 121. The arthrospores appear mainly round with a fibrillar coating and encircling indentations, X 13568.

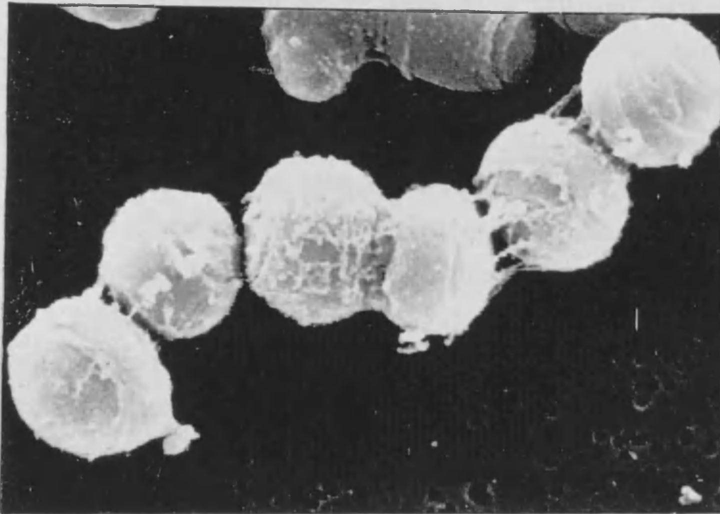


Figure 2:6:- Scanning electronmicrograph of arthrospores of T.mentagrophytes strain 121. Fibrils connecting the arthrospores and fragments of the fibrillar coating are seen, X 11944.

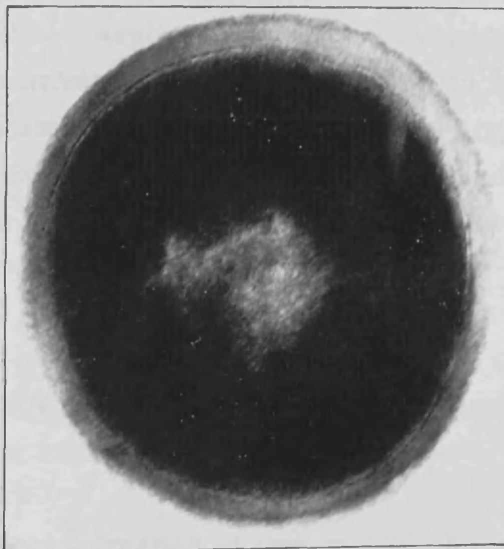


Figure 2:7:- Transmission electronmicrograph of an arthrospore of T.mentagrophytes strain 121. The arthrospore has a thick wall coated with grainy material and a well defined plasma membrane, X 116660.

2:6 Discussion:-

2:6:1 Arthrospore formation at 37°C:-

Dermatophytes in culture grow best at a temperature of 28°C with the exception of T. verrucosum which has an optimal temperature of 37°C when abundant chlamydospores in chains are formed. In this study arthrospore formation was obtained at an incubation temperature of 37°C. Arthrospore formation was also reported in a strain of T. mentagrophytes using various inoculation procedures; membrane sandwich culture (Emyanitoff and Hashimoto, 1979), aqueous microconidial suspension on SDA (Hashimoto and Blumenthal, 1977) and broth overlay on microconidia - inoculated dialysis membrane (Hashimoto and Blumenthal, 1978; Pollack, Lange and Hashimoto, 1983). The temperature of skin where arthrospore formation in dermatophytosis usually occurs has been recorded at a range of 33.2 - 34.4°C (Silberstein, Bahr and Kattan, 1975).

2:6:2 Arthrospore formation in absence of glucose:-

Fungi cannot photosynthesize carbohydrates from water and carbon dioxide in the presence of light because they, unlike plants, lack chlorophyll. They require organic materials to serve both as an energy source and a carbon skeleton for cellular synthesis. In this study when glucose was eliminated from media arthrospore formation occurred in strains of T. interdigitale. Arthrospore formation in strains of T. mentagrophytes has also been reported in media lacking glucose (Emyanitoff and Hashimoto, 1979; Wright et al., 1984). In sweat and in slices of human epidermis glucose has been reported but in very small amounts which were appreciably less than the serum level of glucose (Boysen et al., 1984; Schragger, 1962). The normal serum level of glucose (about 120mg/100ml) is considerably less than the amount normally used in cultures of dermatophytes where arthrospore formation is not usual.

2:6:3 Arthrospore formation in presence of amphotericin-B:-

Presence of amphotericin-B in the growth medium (SDA and SDB) was not associated with arthrospore formation in any of the strains. However, Emyanitoff and Hashimoto (1979) reported formation of arthrospores in T. mentagrophytes at a similar concentration of amphotericin-B and also in

the presence of griseofulvin and clotrimazole at concentrations of 0.5ug/ml and 0.1ug/ml respectively. Perhaps the concentration at which amphotericin-B had been used in this study was low for the tested strains of T. interdigitale. Antifungal drugs generally interfere with growth of fungi in one way or another. Factors which interfere with growth of dermatophytes have been reported in the skin; serum factors(s), sweat and/or its content of ascorbic acid and sebum (Chapter 1, Section 1:5) Furthermore, an inflamed skin is bathed in serum; skin infected with zoophilic and geophilic species of dermatophytes can be highly inflamed.

2:6:4 Arthrospore formation under CO_2:-

The dermatophytes are aerobic microorganisms. Chin and Knight (1957) while studying macroconidial formation under increased CO_2 tension noticed formation of arthrospores in T. rubrum and T. mentagrophytes. King et al.,(1976) identified CO_2 as the factor which was elaborated from Candida species and resulted in inhibition of growth of dermatophytes and arthrospore formation when the two fungi were cocultured under the same atmospheric conditions. In the present study strains of T. interdigitale and T. mentagrophytes produced arthrospores under 10% CO_2. Previous investigators have also reported arthrospore formation in dermatophytes under increased CO_2 tension (Emyanitoff et al., 1979; Weigl and Hejtmanek, 1979 ; 1980; Wright et al., 1984; Fujita et al., 1986). While Emyantoff and Hashimoto, (1979) attributed arthrospore formation under CO_2 at 30°C, to the resulting decrease in oxygen tension because the strain they tested produced arthrospores also under increased nitrogen tension at 30°C, King et al. (1976) reported that the inhibition of growth of dermatophytes occured also with other gases (methane and helium) but arthrospores were formed at 30°C only with CO_2. In like manner, Wright et al. (1984) obtained arthrospores in T. mentagrophytes in the presence of increased tension of CO_2 but not of nitrogen at both 30°C and 37°C. Somehow in contrast to Wright et al (1984), Weigl and Hejtmanek (1980) in a strain of T. mentagrophytes (TM-2) which had not formed arthrospores in control medium incubated at 37°C, obtained arthrospores with CO_2 (8%) at 31°C and 37°C and with nitrogen (8%) but only at 37°C. However, in a later communication Weigl and Hejtmanek. (1980) stated that in strain TM-2 there were arthrospores differentiated also in control media incubated at 37°C. Thus their earlier finding of arthrospores with nitrogen might be at least partially, due to the temperature effect.

From this present and the previously reported studies the effect of CO_2 on arthrospore formation is evident but it is not clear whether this effect is due to a decrease in oxygen tension or a direct link of CO_2 with arthrospore formation. From skin, CO_2 was found to be emitted physiologically at, however, a low rate e.g. from hand 4.6 x 10^{-5}Ml/cm^2/min, but, with hydration e.g. sweating, emission increased (Frame et al., 1972), and when areas of skin were occluded CO_2 made up to 8-10% of the occluded atmosphere (Aly et al., 1978; King et al., 1979). Moreover, a dermatophyte infected skin is a damaged tissue and CO_2 emission from damaged skin has been found to be higher than normal (Malten and Thiele, 1973).

2:6:5 Arthrospore formation in vivo:-

It is clear from the preceding discussion that cultural conditions under which arthrospore formation occurred can be matched to factors present physiologically in the skin and/or become intensified pathologically, for example, as a result of a dermatophytosis. The cutaneous factors if to be compared individually with their in vitro counterparts look generally of a milder nature but this is perhaps compensated for by their combined occurrence in the skin. An ideal medium for the production of arthrospores thus appears to be, one that contains glucose in a limited amount plus some form of growth inhibitory factors and incubated at about 37°C under an atmosphere supplemented with CO_2 at a concentration probably less than 10%. The highest yield of arthrospores in a responding strain has been obtained when of three tested conditions, 37°C, CO_2, elimination of glucose, two were combined, glucose elimination and CO_2, (Wright et al, 1984).

In this study micro and macroconidial and/or chlamydospore formation occurred in cultures of those strains which did not readily form arthrospores or did so mildly. A similar finding had been reported by other investigators (Wright et al., 1984; Weigl and Hetjmanek, 1980; Chin and Knight, 1957). Thus, the cultural conditions examined in this study are not exclusive for arthrospore formation, not like skin where spores other than arthrospores are not produced. This suggests that, in the skin, yet unidentified factor or factors may be involved in arthrospore formation either solely or in combination with others. The role of the in vivo environment in arthrospore formation can be substantiated by the finding in white superficial onychomycosis. In this disease the common causal agent T. mentagrophytes grows on the superficial surface of mostly toenails in a particular morphological form (hyphal fronds) which do not resemble those seen normally in invasion of stratum corneum, hair or even nail in

distal and proximal subungual onychomycosis but rather typical of its culture on a nail (Zaias, 1972;1980). In this superficial location the dermatophyte is very much removed from the host physiological factors. Also an inflammatory reaction which may intensify the host physiological factors had not been observed in white superficial onychomycosis (Zaias, 1966).

2:6:6 Mediation of effect of cultural conditions on arthrospore formation:-

Morphologically, arthrospore formation is entirely different from hyphal growth which consists of extension and development of lateral branches. The original hyphal wall does not become part of the newly deposited wall of arthrospores in T. mentagrophytes but rather shed when arthrospores mature (Hashimoto et al., 1984). Chemically, in T. mentagrophytes arthrospores are different from hyphae. Pollock et al. (1983) reported that after treatment with gluconase and chitinase, the lateral walls of hyphae and most of the septum except for an annulus were completely digested unlike arthrospores in which a portion retaining the original wall shape remained and was present as a continuous layer throughout the arthrospore wall.

The exact manner by which the various cultural conditions affect the formation of arthrospores is not known but it is explicit that they are not deleterious to the process of arthrospore formation per se.

In the present study aseptate hyphae, as well as micro and macroconidia, have been observed in the same media along with arthrospores. This has been reported by other investigators (King et al., 1976; Weigl et al., 1980; Wright et al., 1984). Indeed, in materials from lesions of dermatophytosis, aseptate and septate hyphae along with arthrospores are usually noticed. Onsberg (1979) reported their presence at different degrees of combination in a large amount of material from lesions caused by various dermatophytes. Thus it seems that the two processes, arthrospore formation and hyphal growth, can occur together in a given colony of a dermatophyte either in vitro or in vivo. Complete cessation of hyphal tip elongation has been reported to not be required for the initiation or even progression of arthrosporogenesis when it occurred in terminal hyphal segments (Hashimoto et al., 1984). These investigators also found that arthrospore formation could begin in any segment of a hypha regardless of its position (terminal, subterminal, and intercalary) or its length.

2:6:7 Potential of arthrospore formation under various cultural conditions:-

Differences in the potential of arthrospore formation under CO_2 between individual strains of T. mentagrophytes have been shown by Weigl and Hejtmanek, (1980). In the present study similar differences have been found in strains of T. interdigitale not only with CO_2 but also with other cultural conditions; 37°C and elimination of glucose (Table 2:1). Also differences in the degree of arthrospore formation under the various cultural conditions in the strains of T. interdigitale and under CO_2 in the strains of T. mentagrophytes have been found (Table 2:1).
The reason for these differences has not been researched but may have a genetic basis. Emmons and Hollaender (1939) showed that beside having a lethal effect, ultraviolet irradiation of T. mentagrophytes resulted in a striking production of variants (in the amount of pigment, aerial hyphae and spores). They believed that because of the sudden appearance and permanence, the variants represented true genetic changes (mutation). They also reported that, when spores from an old culture of the original strain were isolated, several mutants spontaneously appeared; some of them were similar to those induced by the ultraviolet radiation. Therefore, they proposed that ultraviolet radiation merely accelerated a process normally operative in these fungi in nature either in the saprophytic phase of the fungus when it occurs or in its parasitic phase in the skin. In agreement with this proposal is the report of Georg, (1954) of the conversion of a granular type culture of T. mentagrophytes (var granulare) into a downy type culture of T. mentagrophytes (var interdigitale) by repeated subculture and of a downy type culture into a granular type culture by serial animal passage. The conversion of the downy type cultures which originally grew as mycelia without hair invasion was associated with hair invasion of the ectothrix type, i.e "arthrospore formation". Thus it seems that the potential of arthrospore formation is present in the dermatophytes but in vitro they require some type of triggering mechanism. This potential has an epidemiological significance as it may help to explain the emergence and disappearance of an outbreak of a dermatophytosis.

2:6:8 Disarticulation of arthrospores:-

Conditions of reduced humidity have been noticed to enhance separation of arthrospores from each other (Hashimoto and Blumenthal, 1977; Wright et al., 1984). In the present study an almost completely disarticulated chains of

arthrospores were obtained with CO_2. This, similarly, may be due to the binding of water molecules by CO_2 thereby reducing humidity. The arthrospores, initially in addition to septal attachment are held to each other by the original hyphal wall but this wall ultimately becomes worn and shed to the exterior (Hashimoto et al., 1984). The fragments on arthrospores and connecting fibrils seen in Figure 2:6 may be remnants of the original hyphal wall. The process which causes the breaking down of the original hyphal wall has not been investigated. As a result of a reduced humidity, however, the original hyphal wall may lose its pliability and become easily cracked by the expansion of the arthrospore which assumes a round appearance (Figure 2:6).

Mechanically, arthrospore expansion at the polar sides can also bring about their separation at the septum. The reason behind arthrospore expansion is not known. Alternatively, an enzymatic action may be operative in the separation of arthrospores and if so it must be well controlled.

2:7 Conclusion:-

Arthrospore formation occurred in vitro under conditions unusual for culturing dermatophytes. These conditions resemble physiological factors present in the skin. With CO_2 an abundant formation of disarticulated arthrospores was obtained. CO_2 therefore was adopted as the standard procedure for the production of arthrospores in the entire study.

Arthrospores are surrounded by thick walls covered with fibrillar material. When disarticulated, arthrospores appear mainly round.

Chapter 3:

Adherence of arthrospores to corneocytes.

3:1 Summary:-

The interaction (adherence) between human corneocytes and arthrospores was studied. Arthrospores of one strain of T. interdigitale and two of T. mentagrophytes, and corneocytes from two skin sites, namely palm and sole, were employed. Adherence of arthrospores to corneocytes occurred and increased with time up to 6 hr by which time germination of arthrospores had started. The interaction between arthrospores and corneocytes was verified by scanning and transmission electronmicroscopy.

3.2 Introduction:-

Adherence of micro organisms to host tissues is now a well established fact. It is regarded as an important means by which microbes colonize a host surface. Candida albicans, for example, has been shown to adhere to mucosal cells (Sanden et al., 1987) and corneocytes (Collins-Lech et al., 1984; Ray, Digre and Payne, 1984).

Unlike candidosis, dermatophytosis is acquired from an exogenous source because dermatophytes are not part of the normal skin flora (Roberts, 1970; Midgley and Clayton, 1972). Arthrospores are the forms likely to play a major role in the spread of dermatophytosis (Chapter 1, Section 1:6). Dermatophytes invade the stratum corneum of the skin, hair and nail. With the exception of white superficial onychomycosis in which nail is invaded directly on its surface, nail and hair are infected from the adjacent stratum corneum (Chapter 1, Section 1:7). Initial contact between arthrospores and stratum corneum thus seems to be an important event in the establishment of skin lesions and initiation of hair and nail infection.

3:3 Aim of the study

The aim of this study was to investigate the initial contact between arthrospores of T. mentagrophytes and T. interdigitale and human corneocytes. The ultrastructural relationship between arthrospores and corneocytes was also investigated.

3:4 Materials and methods:-

3:4:1 Organisms and stock cultures:-

Two strains of T. mentagrophytes (number 121 and 126) and one strain of T. interdigitale (number 4) (Chapter 2, section 2:4:1) were used. Stock cultures of these strains were preserved on silica gel according to the method described by Gentles and Scott (1979). This method consisted of scraping and dispensing a 10-14 day dermatophyte culture into a sterile solution of skimmed milk powder in distilled water and pouring it into cooled sterile silica gel. The suspension was allowed to cool further in ice for about one hour to counteract the heat generated by wetting the gel. The preparation was then left at room temperature for 2-4 days to dry out and finally stored at 4°C. By this method dermatophytes can be stored up to 4 years.

3:4:2 Production of arthrospores:-

Subcultures made from silica gel on Sabouraud's dextrose agar (SDA) were used to produce arthrospores on SDA under 10% carbon dioxide at 28°C as has been described in Chapter 2, Section 2:4:3 and 2:4:4.

3:4:3 Viability of arthrospores:-

Viability of arthrospores was assayed by the methods described by Hashimoto and Blumenthal (1977) with a modification. This method consists of determining the percentage of germ tube formation in a liquid medium. 0.1ml of an arthrospore suspension (1.5×10^6/ml in sterile distilled water) was added to 1ml Sabouraud's dextrose broth and incubated on a rotary shaker (12rpm) at 37°C for 16 hr. Arthrospores were prepared in distilled water using the method described in Section 3:4:4. At the end of the incubation time samples were mounted onto a piece of Steri-Drape (3M Company, Minnesota, U.S.A.) fixed onto a glass slide with its adhesive side uppermost. The tape was heat fixed at 45°C for 10-15 minutes and stained transversely with Periodic acid Schiff (Appendix). This procedure helped to decrease the clumping of arthrospores and gave a better visualization than a direct mount on a glass slide. The percentage of arthrospores with visible germ tubes was determined in triplicate per experiment.

3:4:4 Preparation of arthrospores:-

Arthrospores were harvested by scraping the culture with a sterile surgical scalpel. They were suspended in phosphate-buffered saline (PBS), (Appendix) and shaken for 5 minutes. The suspension was filtered through column chromatography grade glass wool packed in a 10ml syringe barrel to a depth of 1cm to remove the unseparated chains of arthrospores. The filtered arthrospore suspension was washed three times in PBS at 300g for 3 minutes. The pelleted arthrospores were resuspended in PBS and adjusted to a concentration of 1×10^7/ml.

3:4:5 Preparation of corneocytes:-

Corneocytes were obtained from a healthy volunteer by scraping with a surgical scalpel. To mimick the in vivo situation, for example, microbial interaction and competition for an adherence site by minimizing the removal of the normal skin flora which is likely to occur by the subsequent steps of this procedure, no prior antiseptic swabbing of the area to be scraped was done. Scraped corneocytes were suspended in PBS. It was noticed that if the area to be scraped was first made wet by the corneocyte suspending solution (PBS) the loss of corneocytes which is likely to be single or in small sheets by air dispersal is minimized. Also helping in obtaining single cell suspension is the scraping of a very small sheet at a time. PBS-suspended corneocytes were vigorously shaken until a predominantly single cell suspension was obtained. They were then washed in 10ml PBS three times and adjusted to a concentration of 1×10^6/ml. By this procedure corneocytes from palm and sole were prepared.

3:4:6 Adherence assay:-

The assay described in studies of adherence of Candida albicans to human mucosal cells and corneocytes was used with a modification in the evaluation of adherence. 0.3ml of an arthrospore suspension (1×10^7/ml) was mixed with 0.3ml of a corneocyte (1×10^6/ml) suspension in a rotary shaker (12rpm) at 37°C for various time intervals of 1, 2, 4 and 6 hr. At the end of each time the corneocyte - arthrospore mixture was filtered and washed with 30ml PBS through a 12um polycarbonate capillary pore membrane (Nucleopore) using an automatic suction pump to remove the excess non-adherent arthrospores. The polycarbonate membrane was rinsed in 0.5ml PBS to dislodge its retentate of arthrospore-corneocyte mixture.

From the wash microscopical mounts were made and the total number of arthrospores adhering to 100 corneocytes was counted in triplicate per experiment. Sometimes arthrospores in clumps were seen adhering to corneocytes making accurate quantitation difficult. In such situations the adhering arthrospores were scored as three.

3:4:7 Electronmicroscopy of arthrospore adherence to corneocytes

Standard methods of preparing materials for scanning and transmission electronmicroscopy were used for processing corneocyte-arthrospore mixtures incubated at various time intervals for ultrastructural studies.

3:4:8 Statistical analysis of results:-

Statistical analysis of the results was carried out using the Student's t-test.

3:5 Results:-

3:5:1 Viability of arthrospores:-

An arthrospore was considered to have germinated when a visible germ tube had developed. The viability of arthrospores as has been calculated from three assays of both strains for T. mentagrophytes was 96.00±0.81 and for T. interdigitale was 97.00±1.05.

3:5:2 Adherence of arthrospores to corneocytes:-

Adherence of arthrospores to corneocytes occurred and increased with time up to 6 hr (Table 3:1) by which time germination of arthrospores had started. Arthrospores adhered to the margins and surfaces of corneocytes singly, in pairs, or in clusters (Figure 3:1-3). Statistically significant differences at 6 hr, with palm corneocytes were found between strain numbers 121 and 126, P was exceedingly less than 0.001 and between strain 121 and 4, P was less than 0.05 and with sole corneocytes were found between strain 121 and 4 P was exceeding less than 0.001 and between 126 and 4 P was less than 0.05. In their adherence to palmar and plantar corneocytes at 6hr only arthrospores of strain 126 showed a statistically significant difference, P less than 0.02. Not all corneocytes from both palm and sole had adherent arthrospores. and in

Strain	Time	Palm	Sole
121	1st hour	11 ± 1.49	18.33 ± 4.02
	2nd hour	50.55 ± 6.84	29.11 ± 3.84
	4th hour	58.33 ± 9.96	53.11 ± 7.53
	6th hour	60.88 ± 9.04	63.33 ± 9.01
126	1st hour	10.60 ± 4.16	14 ± 3.94
	2nd hour	20.77 ± 7.53	26 ± 5.27
	4th hour	30.66 ± 6.54	30.88 ± 9.50
	6th hour	34.33 ± 8.95	54.66 ± 16.61
4	1st hour	8.66 ± 3.94	7.33 ± 5.20
	2nd hour	12.88 ± 6.59	10.44 ± 4.37
	4th hour	26.11 ± 10.91	25.22 ± 6.81
	6th hour	42.77 ± 17.59	34.88 ± 9.21

Table 3:1: - Adherence of arthrospores to corneocytes. Data are the mean ± SD of arthrospores adhering to 100 corneocytes and were calculated from three adherence assays; each assay was read in triplicate.

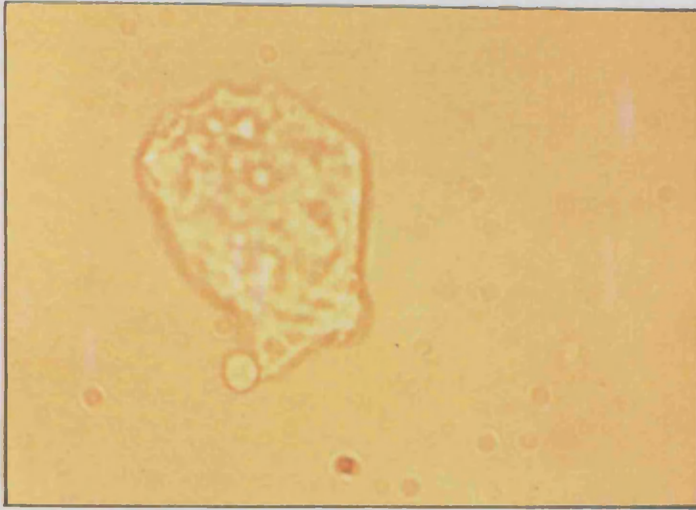


Figure 3:1:- Arthrospore adhering to the margin of a corneocyte, 4 hr incubation, X 708.

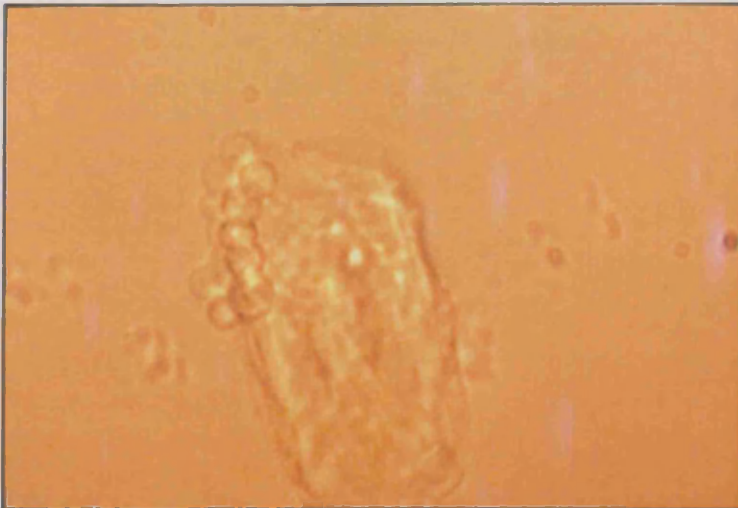


Figure 3:2:- A cluster of arthrospores adhering to the margin of a corneocyte , 4 hr incubation, X 708

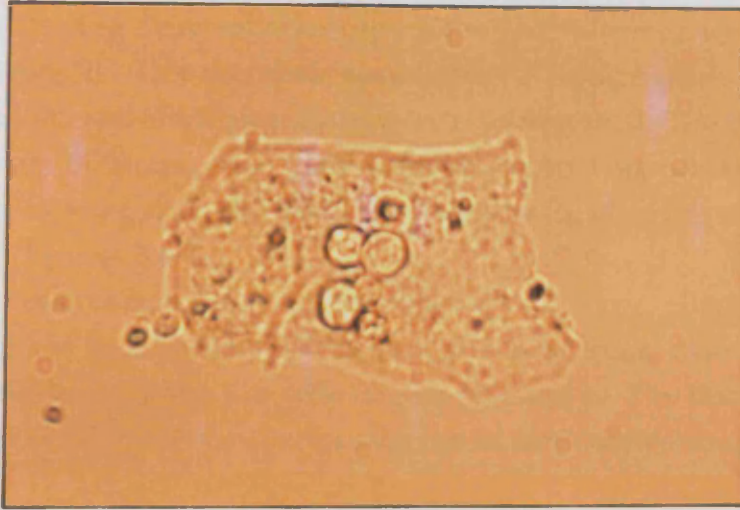


Figure 3:3:- A cluster of arthrospores adhering to the surface of a corneocyte, 6 hr. incubation, X 708

Table 3:2 corneocytes with adherent arthrospores is shown, there it can be seen that there is also a time dependent increase.

3:5:3 Ultrastructure of adherence of arthrospores to corneocytes:-

Under scanning electronmicroscopy (SEM) arthrospores were seen attached to corneocytes. The roundish appearance of arthrospores with encircling indentations and fibrillar coating were also observed. The corneocytes were hexagonal in shape and their surfaces were convoluted. Arthrospores adhered to more than one fold. They were seen adhering singly, in pairs or clusters (Figures 3:4-7).

With transmission electron microscopy a very close apposition of arthrospores to corneocytes was seen and a floccular material appeared to bridge the walls of the two cells (Figure 3:8a and b). The thick cell wall of the arthrospores and the keratinous skeleton of corneocytes could also be seen.

3:6 Discussion:-

3:6:1 Adherence of arthrospores to corneocytes:-

This study showed that arthrospores of a zoophilic dermatophyte, T. mentagrophytes, and an anthropophilic dermatophyte, T. interdigitale, adhered to human corneocytes from the palm and sole. Recently, adherence of arthrospores and microconidia of dermatophytes to corneocytes has been reported (Zurita and Hay, 1987).

In the present study arthrospore adherence to corneocytes increased with time up to 6 hr. Similarly, adherence of Candida albicans to corneocytes was reported to show a time dependent increase (Ray et al., 1984).

By the 6 hour of incubation germination had started and both adherent and non adherent arthrospores germinated. In the case of C. albicans germination has been reported to enhance its adherence to buccal and vaginal mucosal cells (Kimura and Pearsall, 1980; Sobel et al., 1981). The effect of germination on the adherence of arthrospores to corneocytes needs further evaluation. However, it was found in the present study that at 2 and 4 hr when germination had not been observed, levels of adherence were sometimes comparable to those seen at 6 hr (Table3:1). Before the emergence of germ tubes, fungal spores in general are known to undergo an increase in size (swelling) as a result of intake of water and increases in respiration and

Strain	Time	Palm	Sole
121	1st hour	8.66 ± 1.56	10.33 ± 2.00
	2nd hour	20.77 ± 2.39	15.44 ± 3.33
	4th hour	24.11 ± 2.72	26.88 ± 4.79
	6th hour	23.33 ± 3.36	30.22 ± 6.51
126	1st hour	7.33 ± 2.98	9.22 ± 2.89
	2nd hour	10.88 ± 5.02	11.22 ± 2.48
	4th hour	12.44 ± 2.11	14.66 ± 6.71
	6th hour	11.88 ± 2.51	20.66 ± 6.68
4	1st hour	6.4 ± 1.94	5.11 ± 3.07
	2nd hour	7.2 ± 2.43	6.22 ± 1.74
	4th hour	13.50±5.83	16.33 ± 3.91
	6th hour	21.33±5.24	19.77 ± 3.67

Table 3:2: - Corneocytes with adhering arthrospores. Data are the mean ± SD of percentages of corneocytes with adhering arthrospores and were calculated from three adherence assays; each assay was read in triplicate.

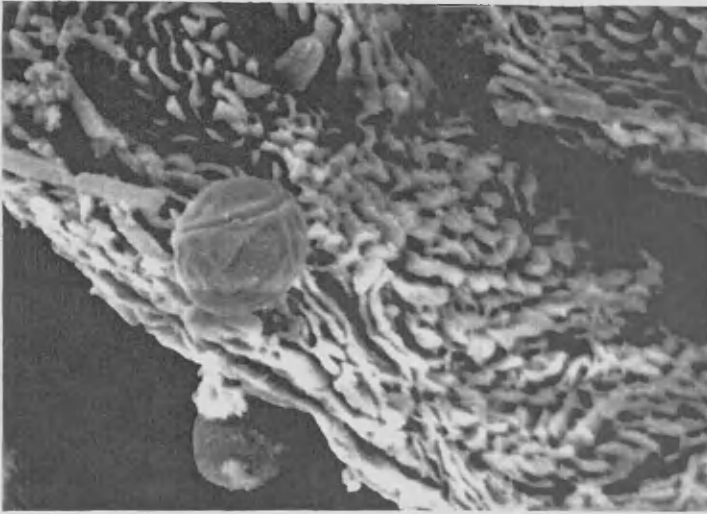


Figure 3:4:- Scanning electronmicrograph of arthrospores adhering singly to the margin of a corneocyte 4 hr incuabtion. Bacteria are seen also adhering to the corneocyte (arrows), X 8704.

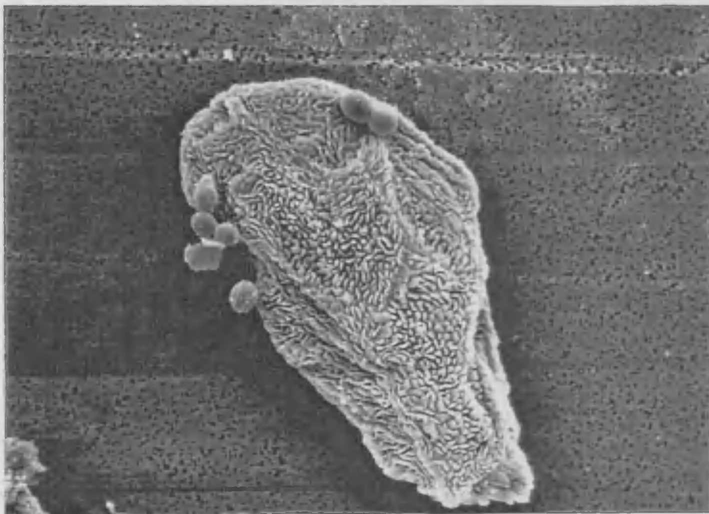


Figure 3:5:- Scanning electronmicrograph of arthrospores adhering singly, in pairs and in clusters to the margin of a corneocyte, 6 hr incubation. Arthrospores had started to germinate (arrow), X 2176.

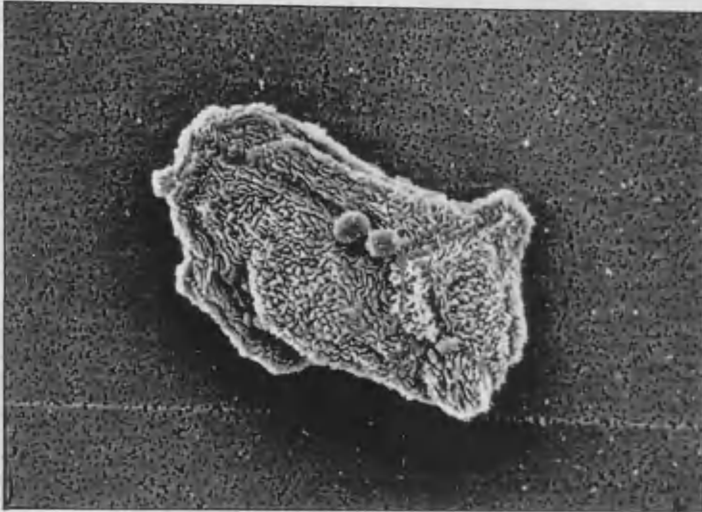


Figure 3:6:- Scanning electronmicrograph of arthrospores adhering to the surface of a corneocyte, 4 hr incubation. A bacterium is seen also coadhering to the arthrospore (arrow), X 2176.

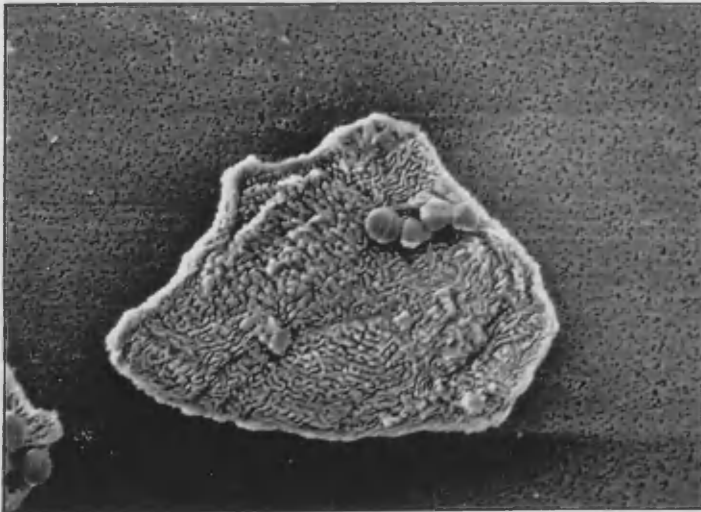


Figure 3:7:- Scanning electronmicrograph of arthrospores adhering to the surface of a corneocyte, 6 hr incubation. Arthrospores had started to germinate (arrow), X 2176.

Figure 3:8a

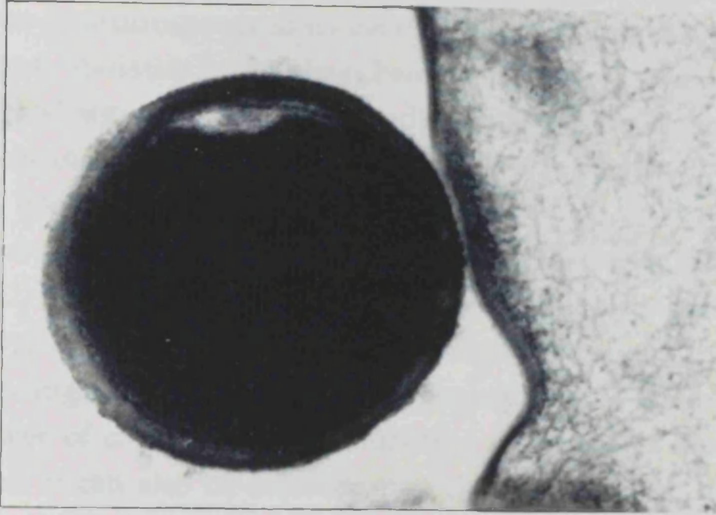


Figure 3:8b

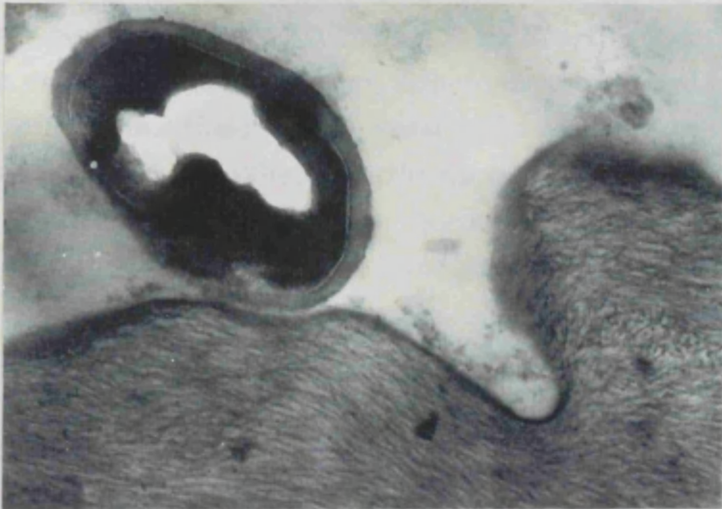


Figure 3:8:- Transmission electronmicrographs (a and b) of arthrospores adhering to corneocytes. A floccular material is seen bridging the closely apposed walls of the arthrospores and corneocytes, a, 6 hr incubation; b, 4 hr incubation, a X 65750, b X 83250.

in biosynthesis of many cell components (Hawker, 1966, Gottlieb, 1966). However, the effect of these changes on arthrospore adherence to corneocytes is not understood but it is known that arthrospores of dermatophytes also undergo swelling before the emergence of germ tubes (Scott, Gorman and Wright, 1984). Quantitative differences on the adherence of arthrospores to corneocytes were found and in occasions were significant statistically as has been mentioned in section 3:5:2. The pathological significance of this finding is not fully clear; it may indicate differences in adherence rates of various dermatophytes to corneocytes from different body sites.

The number of corneocytes with adherent arthrospores increased with time (Table 3:2) and in Table 3:3 the average number of arthrospores adhering per corneocyte is shown and this also increased with time. This increase might reflect an augmentation of the adherence capacity of the arthrospores. Since the number of corneocytes with adherent arthrospores increased with time (Table 3:2) it can also be assumed that changes occurred to the corneocytes which increased their arthrospore binding capacity.

3:6:2 Ultrastructure of arthrospore adherence to corneocytes:-

Ultrastructurally, floccular material was seen at the sites where arthrospores adhered to corneocytes (Figure 3:7a and b). The origin of this material is not clear, but it might be of arthrospore origin, i.e. from the fibrillar coating of the arthrospore (Figure 3:4-7; Chapter 2, Section 2:5:8), or from the stratum corneum or both. With C. albicans, adherence to mucosal cells is thought to be mediated by an adhesin on the yeast and a receptor on the cell, (Critchley and Douglas, 1987a,b). Whether arthrospore adherence to corneocytes was mediated specifically by an adhesin-receptor mechanism or non-specifically, e.g. ion-bridging or electrostatic force has not been studied.By scanning electronmicroscopy, arthrospores could be seen adhering to more than one convolution of a corneocyte thereby confirming a firmer attachment.

3:6:3 Significance of arthrospore adherence to corneocytes:-

Because arthrospores are regarded to play a major role in the spread of infection, the finding in this study of arthrospore adherence to human corneocytes denotes a pathological importance. The ability of microorganisms to adhere to host tissues serves generally two purposes. Firstly, it prevents detachment from host surfaces, for example, by mucosal secretion as in the case of C. albicans colonization of the oral cavity and by

Strain	Time	Palm	Sole
121	1st hour	1.27 ± 0.95	1.77 ± 2.01
	2nd hour	2.43 ± 2.86	1.88 ± 1.15
	4th hour	2.41 ± 3.66	1.97 ± 1.57
	6th hour	2.60 ± 2.69	2.09 ± 1.38
126	1st hour	1.44 ± 1.39	1.51 ± 1.36
	2nd hour	1.90 ± 1.5	2.31 ± 2.12
	4th hour	2.46 ± 3.09	2.10 ± 1.41
	6th hour	2.88 ± 3.56	2.64 ± 2.48
4	1st hour	1.35 ± 2.02	1.43 ± 1.69
	2nd hour	1.23 ± 2.71	1.67 ± 2.51
	4th hour	1.93 ± 1.87	1.54 ± 1.74
	6th hour	2.00 ± 3.35	1.76 ± 2.50

Table 3:3: - Arthrospores adhering per corneocyte. Data were calculated by dividing the mean and SD of arthrospores adhering to 100 corneocytes (Table 3:1) by mean and SD, respectively, of corneocytes with adhering arthrospores (Table 3:2).

urinary flow in the case of Escherichia coli infection of the urinary tract (Eden et al., 1988). Secondly, it promotes multiplication on host surfaces and delivery of toxins. Arthrospores are neither known to be mucosal colonizers nor deliver toxins . In this study, germination of arthrospores in corneocyte suspensions occurred after 6 hr. Therefore, the pathological significance of adherence of arthrospores to corneocytes is by enabling them to remain on the stratum corneum and develop into invasive forms, hyphae, they establish an infection. Alternatively in the establishment of dermatophytosis the primary event may be corneocyte to corneocyte adherence, i.e. an infected corneocyte(s), with arthrospore(s) adhering to it, adheres to the stratum corneum of a new host. Clearly, if operative, this possibility is dependent on the adherence of arthrospores to the infecting corneocytes(s). In both cases the adherence of arthrospores to corneocytes is an important event and can be regarded as a pathogenic factor in dermatophytosis.

Finally, the crevices which are present in the outer aspect of the stratum corneum may, by trapping arthrospores, play a role in their adherence to corneocytes.

3:7 Conclusion:-

Adherence of arthrospores of dermatophytes to human corneocytes occurred. The adherence showed a time dependent increase up to 6 hr by which time germination had started. Ultrastructurally, the adherence manifested as a very close apposition between the arthrospores and the corneocytes. A floccular material connected the walls of the two cells. The origin of this material is not known but possibilities have been mentioned. The significance of the findings in relation to the pathogenesis of dermatophytosis has been discussed.

Chapter 4:

Germination of arthrospores in corneocyte suspensions.

4:1 Summary:-

Germination of dermatophyte arthrospores in suspensions of human corneocytes was investigated. Arthrospores of T. mentagrophytes and T. interdigitale and corneocytes from palms and soles were used. After 6 hr incubation germination had started and increased with time up to 16 hr when long branched germ tubes were seen. Ultrastructural studies showed that germ tubes penetrated through corneocytes. Pretreatment of arthrospores with distilled water at 28°C for 24 hr enhanced their germination. The pathological significance of these findings is discussed.

4:2 Introduction:-

Germination is the first step in the development of fungal mycelium from a spore. It has been defined in different ways. Sussman (1966) defined it as the first irreversible stage which is recognisably different from the dormant organism as judged by physiological or morphological criteria and Manners (1966) defined it as a process that includes internal changes, morphological and physiological changes within the confines of the spore wall; germination, the act of protrusion of the germ tube from the spore wall and germ tube growth, the elongation of germ tube. Morphologically, there is an increase in the size of the spore which sometimes is accompanied by an alteration of shape (Hawker, 1966). Physiologically, there are increases in respiration and in biosynthesis of many cell components (Gottlieb, 1966).
Some of the fungi are pathogenic to man, animals and plants but many exist only as saprophytes in nature. Accordingly, the nutritional and physical requirements for germination vary among fungal spores. Hashimoto and Blumenthal, (1977) studied the germination of T. mentagrophytes arthrospores in a variety of laboratory media under different conditions. They reported that these spores germinated readily in rich complex media such as Sabouraud's dextrose broth but very poorly (10%) in the presence of single amino acids, simple peptides, distilled water or sodium phosphate buffer. When arthrospores were pretreated by incubation in distilled water at 25°C for 24 hours the poor germination rate was increased.

4:3 Aim of the study:-

The aim of the study was to investigate the ability of dermatophyte arthrospores to develop in human corneocytes. Additionally, the ultrastructural relationship between the growing arthrospore and corneocyte was studied. Because corneocytes are the cells which constitute the target tissue of dermatophytes (stratum corneum), this study may help to understand the kinetics of their invasion of this tissue.

4:4 Materials and Methods:-

4:4:1 Organism and stock cultures

The organisms employed in this study and the stock cultures were the same as described previously in Chapter 3, Section 3:4:1.

4:4:2 Preparation of arthrospores:-

Arthrospores were produced, prepared and adjusted to a concentration of 1 x 10^7/ml in PBS in the manner described in Chapter 3, Sections 3:4:2 and 3:4:4.

4:4:3 Viability of arthrospores:-

The viability of arthrospores was assessed by the method described in Chapter 3, Section 3:4:3.

4:4:4 Preparation of corneocytes:-

Human corneocytes from the palm and sole were prepared and adjusted to a concentration of 1 x 10^6/ml in PBS as described in Chapter 3, Section 3:4:5.

4:4:5 Germination assay:-

0.3ml of each corneocyte preparation and freshly harvested arthrospore suspension in PBS were tumble mixed (12 rpm) at 37°C for 6, 8, 16 and 21 hr. As a control, 0.3ml of the arthrospore suspension was incubated in 0.3ml of either Sabouraud's dextrose broth or PBS in a similar manner for the various times. At the end of each time period samples were mounted and

spread on a piece of Steri Drape fixed on a glass slide with its adhesive side uppermost. Tapes were heat fixed at 45°C for 10-15 minutes and stained with periodic acid-Schiff (Appendix). The percentage of germination (formation of a visible germ tube) was determined in triplicate per experiment. Each experiment was performed three times.

4:4:6 Effect of exposure of arthrospores in distilled water at 28°C for 24 hr on germination:-

The methods described previously (Chapter 3, Sections 3:4:2 and 3:4:4 were used to harvest and prepare arthrospores in distilled water to a final concentration of 1 x 10^7/ml. Suspensions of arthrospores in distilled water were then incubated at 28°C for 24 hr. Arthrospores germination was determined at the end of this period and suspensions were then centrifuged and arthrospores were resuspended in PBS. The concentration of arthrospores was readjusted to 1 x 10^7/ml. Assessment of germination of arthrospores was carried out using the method described in the previous section (4:4:5).

4:4:7 Electronmicroscopy of arthrospore germination in corneocyte suspensions:-

Samples from the germination assay were processed for both scanning and transmission electronmicroscopy using standard methods. The studied samples were those of arthrospores of T. mentagrophytes strain 121 grown in suspensions of corneocytes from sole.

4:4:8 Statistical analysis of results:-

Statistical analysis of the results was carried out using the student's t-test.

4:5 Results:-

4:5:1 Viability:-

An arthrospore was considered to have germinated when a visible germ tube has developed. The viability of arthrospores as calculated from 3 assays

was for strain 121, 96.00 ± 1.63, strain 126, 97.00 ± 1.24 and strain 4, 95.00 ± 1.76.

4:5:2 Germination of arthrospores:-

By 6 hr, germination had started and increased with time up to 16 hr (Table 4:1) by which time long germ tubes had developed (Figure 4:1). Multiple germination of arthrospores and branching of germ tubes were observed (Figure 4:2 and 4:3). Adherent and non adherent arthrospores had germinated and sometimes germ tubes were seen adhering to corneocytes (Figure 4:1 and 4:2). In Sabouraud's dextrose broth (SDB), germination of arthrospores similarly had occurred by 6 hr but at a rate higher than that seen in corneocyte suspensions, and it increased rapidly with time (Table 4:1). In PBS, germination of arthrospores had occurred but it was of a low level (Table 4:1). At 16 hr a statistically significant difference between the germination of arthrospores in corneocytes from palm and sole was found only for strain 121, P less than 0.01. The difference between the germination of arthrospores of strain 126 and 4 in corneocytes from palm at 16 hr was significant, P less than 0.01 and in corneocytes from sole at 16 hr the differences between 121 and 4, on one hand, and 121 and 4, on the other hand, were also significant, P less than 0.01.

4:5:3 Effect of exposing arthrospores in distilled water at 28°C for 24 hr on germination:-

Essentially, no germination was observed after exposure in distilled water at 28°C for 24 hr. The germination of these exposed arthrospores in suspensions of corneocytes is shown in Table 4:1. In all the strains at 6 and 8 hr it is significantly higher than that of unexposed arthrospores, P values were less than 0.01. At 16 hr significant differences, P less than 0.02, were found between the germination of the exposed and unexposed arthrospores of strain number 4 in the sole corneocyte suspensions and of both 121 and 126 in the palm corneocyte suspensions. In PBS, germination of exposed arthrospores of all strains was greater than that of unexposed arthrospores. However, the germination remained lower than that seen in corneocyte suspensions at the corresponding time of incubation even with unexposed arthrospores (Table 4:1).

Strain	Time	Arthro spore	Palm	Sole	PBS	SDB
121	6th hour	Fresh	12.55±2.98	20.77±7.05	3.44±1.70	46.66±3.39
		Exposed	32.33±3.29	32.00±3.77	6.33±1.24	ND
	8th hour	Fresh	23.00±5.14	23.77±9.60	6.00±3.46	65.00±4.80
		Exposed	43.66±5.55	50.11±11.70	12.77±5.37	ND
	16th hour	Fresh	54.55±3.88	63.66±5.07	16.55±3.37	89.44±3.56
		Exposed	63.11±2.68	64.00±5.73	18.33±2.35	ND
126	6th hour	Fresh	18.22±3.04	7.11±1.36	1.22±1.33	39.77±4.89
		Exposed	41.00±5.33	34.77±4.15	8.22±1.31	ND
	8th hour	Fresh	23.00±5.03	14.11±2.68	5.55±2.75	62.66±2.21
		Exposed	45.11±5.19	30.66±15.24	14.80±5.42	ND
	16th hour	Fresh	59.22±5.15	55.88±8.47	10.11±3.44	87.88±4.43
		Exposed	65.44±4.32	58.55±4.87	18.66±5.41	ND
4	6th hour	Fresh	9.33±3.43	8.11±1.96	2.11±1.59	36.11±4.65
		Exposed	36.55±12.48	26.11±4.17	4.00±0.81	ND
	8th hour	Fresh	31.11±5.30	15.33±6.21	5.00±0.94	54.44±4.87
		Exposed	48.77±7.13	38.33±6.74	7.22±2.24	ND
	16th hour	Fresh	49.66±5.77	55.11±4.09	7.33±2.30	87.66±3.94
		Exposed	55.77±5.84	65.22±6.71	10.55±4.34	ND

Table 4:1: - Germination of arthrospores in corneocyte suspensions. Data are the mean ± SD of percentages of germinated arthrospores and were calculated from three germination assays; each assay was read in triplicate.

ND = not done

Fresh = Freshly harvested arthrospores

Exposed = Arthrospores which were exposed in distilled water at 28°C for 24 hr.

Figure 4:1:- Arthrospore germination in corneocyte suspension, 16 hr incubation. Germ tube of free arthrospore (not adhering to corneocyte) is seen adhering to corneocyte (arrow), X 252.

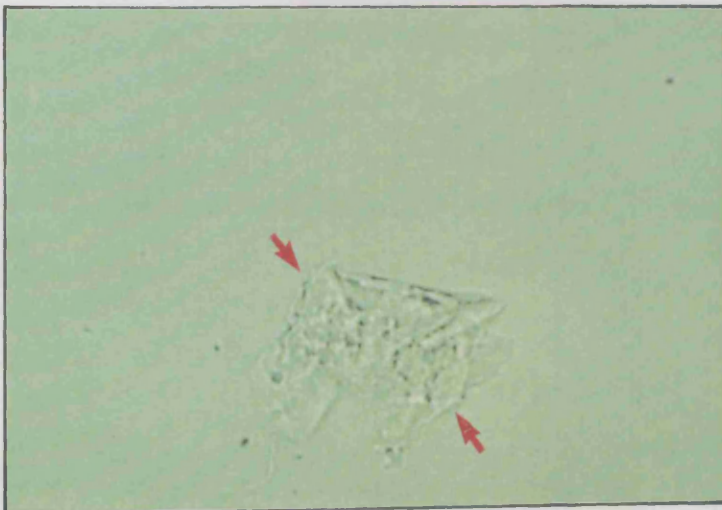
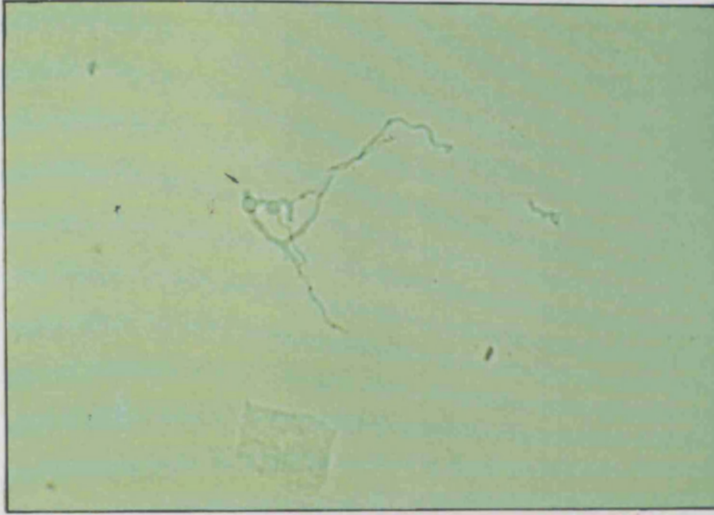


Figure 4:2:- Arthrospore germination in corneocyte suspension, 21 hr incubation. Germ tubes are seen multibranched and adhering to corneocytes (arrows), X 252.



<u>Figure 4:3:-</u> Arthrospore germination in corneocyte suspension, 21 hr incubation. Free arthrospores (not adhering to corneocyte) are seen multigerminated with germ tubes multibranched, X 252.

4:5:4 Ultrastructure of arthrospore germination in corneocyte suspensions:-

Protruding small germ tubes were observed in corneocyte suspensions after 6 hr incubation by both scanning (SEM) and transmission electronmicroscopy (TEM) (Figure 4:4 and Figure 4:5). With SEM small germ tubes could be seen either lying close to the surface of corneocytes; or directed away from them. In TEM the wall of arthrospores could be seen reduced in thickness over the protruding tubes which were filled with influx from the arthrospore cytoplasm. In samples after 21 hours incubation examined with SEM (Figure 4:6-8) germ tubes were seen penteterating into corneocytes which appeared ragged.

4:6 Discussion

4:6:1 Growth of arthrospores in corneocyte suspensions:-

It became clear that human corneocytes can trigger the germination of arthrospores; both adherent and non adherent arthrospores had germinated. The germination of arthrospores, both adhering and non adhering to corneocytes, occurred while they were lying outside corneocytes indicating that, either a germination promoting factor(s) had diffused from corneocytes or was present outside, i.e. in the intercellular space. The intercellular space is rich in neutral lipid (Elias, 1987). Processing of corneocytes for these experiments was carried out in PBS and no antiseptic measures were applied to the skin before the corneocytes were scraped off. Thus it is likely that corneocytes have retained some of their lipid coating. The interior of the corneocytes is filled with keratinous material in addition to the various protein markers of cornification and breakdown products of nuclei and cytoplasmic organelles (Chapter 1, Section 1:3). Other factors which might have influenced the germination of arthrospores are incubation temperature of 37°C and hydration. The optimal temperature for arthrospore germination was reported to be 37°C (Hashimoto and Blumenthal, 1977). Hydration (water) is regarded as the prime factor in any germination medium (Gottlieb, 1950). Arthrospore germination in PBS suggests that the arthrospores contain an endogenous source of nutrients. Lipid and glycogen inclusions in hyphae during formation of arthrospores have been shown (Bibel et al., 1977). Chin and Knight (1963) reported that

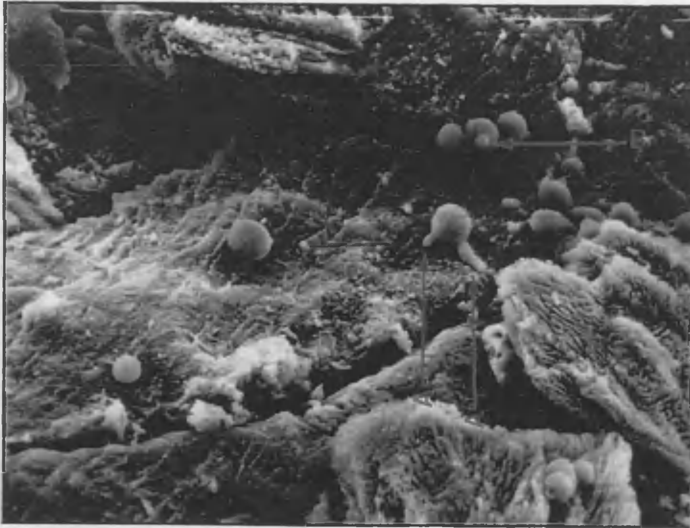


Figure 4:4:- Scanning electron photomicrograph of arthrospore germination in corneocyte suspension, 6 hr incubation. Germ tubes are seen appressed over the surface of the corneocyte (arrow A) or directed away from it (arrow B), X 2176.

Figure 4:5:- Transmission electronmicrograph of arthrospore germination in corneocyte suspension, 6 hr incubation. The thickness of the wall is reduced over the protruding germ tube which appeared to be filled with influx from the arthrospore cytoplasm, X 37 500.

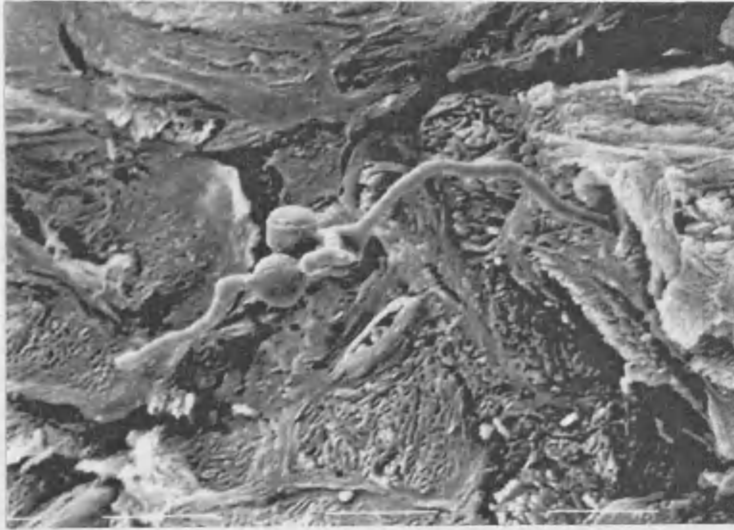


Figure 4:6:- Scanning electronmicrograph of arthrospore germination in corneocyte suspension, 21 hr incubation. A multibranched germ tube (arrow A) is seen penetrating a corneocyte (arrow B), X 2176.

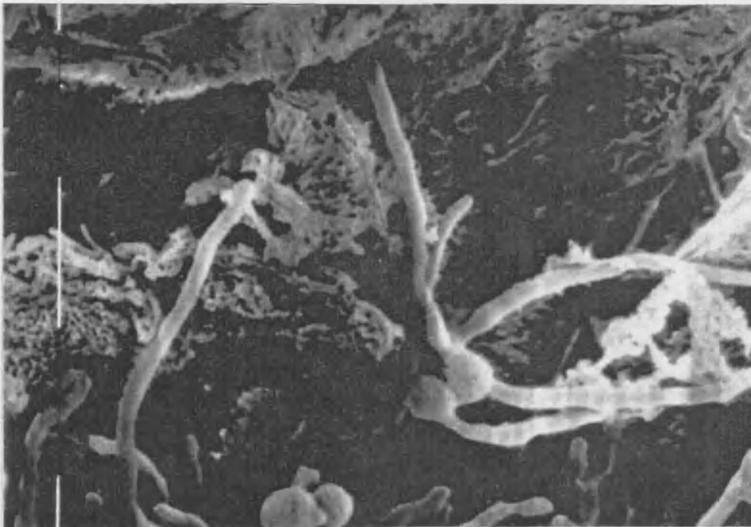


Figure 4:7:- Scanning electronmicrograph of arthrospore germination in corneocyte suspension, 21 hr incubation. Germ tubes are seen penetrating the corneocytes which appear ragged (arrows), X 3040.

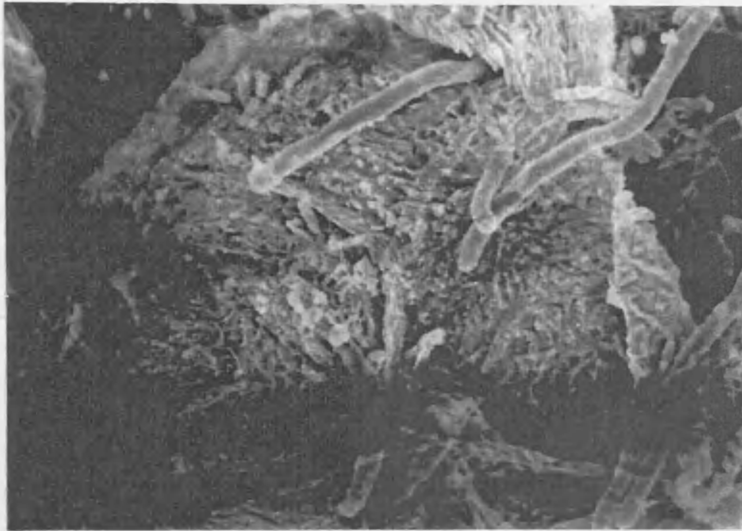


Figure 4:8:- Scanning electronmicrograph of arthrospore germination in corneocyte suspension, 21 hr incubation. The germ tubes are seen emerging from the corneocyte (arrows). The arthrospores from which these germ tubes have developed were likely to be behind the corneocyte, X 4352.

macroconidia, but not microconidia, of Microsporum fulvum and T. mentagrophytes germinated in distilled water and they postulated that the higher concentration of carbohydrate in macroconidia may be more readily available as an energy source for germination than the lower quantity in microconidia. In the present study germination of arthrospores in corneocyte suspensions was, however, far greater than in PBS casting no doubt on the capability of dermatophytes arthrospores to germinate readily in human corneocytes. Moreover, corneocyte suspensions supported further the growth and branching of germ tubes as was observed after 16 and 21 hr incubation (Figure 4:1-4). This may be related to dermatophytes being equipped with a proteo and lipolytic enzyme system (Chapter 1, Section 1:5).The time dependent increase in the germination of arthrospores shows that, the periods required by individual arthrospores to germinate is not equal. The reason for this is not fully understood but may relate to the morphological and physiological changes, mentioned above (Section 4:2), which associate the germination process. Ultrastructurally the corneocytes were penetrated with germ tubes and they appeared ragged. The mechanism by which germ tubes rupture the corneocyte membrane (Figure 4:6-8) is not understood clear but may be caused by enzymatic activity of the dermatophytes or mechanically by the elongating germ tube. This point will be discussed further in Chapter 5.

4:6:2 Significance of arthrospore germination in human corneocyte suspensions:-

In the previous study (Chapter 3) adherence of arthrospores to human corneocytes was found to occur and in the present study germination of arthrospores occurred in suspensions of human corneocytes. Small germ tubes were sometimes seen closely apposed to the surface of corneocytes (Figure 4:5) thus helping to hold the whole structure in position. Long germ tubes adhered to corneocytes (Figure 4:1-3) and ultrastructurally, penetrated into them (Figure 4:7-9). Thus it seems that although adherence of arthrospores to corneocytes occur, germination is a crucial factor in the establishment of a lesion. Furthermore, on the skin if arthrospores adherent to corneocytes fail to germinate they will not produce a lesion because they ultimately will be shed from the surface of the stratum corneum by the skin exfoliative process. Germination of arthrospores therefore should be regarded as a pathogenic (virulence) factor in dermatophytosis. This is further supported by the finding of statistically significant differences (Section 4:5:2) in the germination of arthrospores of the different strains.

The enhanced germination rate of arthrospores after hydration has an epidemiological significance because arthrospores are found in wet areas such as floors of sport centres and communual bathing places or in wet items such as used towels or trunks. Arthrospores are therefore subjected to moist conditions at least in their immediate microenvironment. These places and items are known to act as means for the spread of dermatophytosis. The initial hydrated state will increase the number of germinating arthrospores thereby increasing the chances of causing an infection in new hosts.

4:7 Conclusion

Arthrospore germination in suspensions of human corneocytes occurs and germ tubes penetrate corneocytes. These findings substantiate the role played by arthrospores in the spread of dermatophytosis. Germination of arthrospores can be regarded as a pathogenic factor in dermatophytosis.

Chapter 5

Growth of dermatophytes on stratum corneum

5:1 Summary:-

Growth of T. interdigitale and T. mentagrophytes on stripped sheets of stratum corneum from different body areas was studied.
Arthrospores started germination by 4 hr. With further incubation arthrospore germination increased and germ tubes extended across the stratum corneum. Cross sections of infected stratum corneum showed hyphae growing transversely in and through the thickness of the stratum corneum. By 7 days incubation hyphae started to form arthrospores thereby completing the vegetative growth cycle of the fungus. Scanning electronmicroscopy revealed penetration of corneocytes by germ tubes and the formation of tunnels in the stratum corneum as a result of fungal growth and showed the corneocytes to be damaged after 7 days incubation.

5:2 Introduction:-

Adhesive tapes and cyanoacrylate contact cement have been used to sample lesions of dermatophytosis (Knudsen, 1974; Whiting and Bisset, 1974; Lachapelle et al., 1977). Marks and Dawber (1972) termed stripping the stratum corneum by cyanoacrylate adhesives as skin surface biopsy and regarded it suitable for the investigation of the biology of infection of the stratum corneum. Knight (1972) studied growth of dermatophyte microconidia using sheets of stratum corneum stripped by an adhesive tape. Because 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 dermatophytosis is appropriate.

5:3 Aim of the study:-

Based on the findings of the previous two studies, (Chapter 3 and 4), of arthrospore adherence to corneocytes and germination in suspensions of these cells along with penetration of corneocytes by the germ tubes this study was designed to follow the growth of arthrospores on stripped sheets of stratum corneum. This study may give a view better than that of the

germination assay with regard to the dermatophytes invasion of stratum corneum because the structural integrity of this tissue is maintained. The intimate relationship between the dermatophytes and the corneocytes was investigated ultrastructurally and compared to that in scale from naturally infected skin.

5:4 Materials and methods:-

5:4:1 Organisms and stock cultures:-

The three dermatophytes used previously, namely two strains of *T. mentagrophytes* (121 and 126) and one of *T. interdigitale* (4), were employed in this study. Stock cultures have been described in Chapter 3, Section 3:4:1.

5:4:2 Production and preparation of arthrospores:-

Arthrospores were produced and prepared to a concentration of 1 x 10^7/ml in PBS as described in Chapter 3, Section 3:4:2.

5:4:3 Viability of arthrospores:-

The viability of arthrospores was checked by the method described in Chapter 3, Section 3:4:3.

5:4:4 Preparation of stratum corneum sheets:-

By repeated application to skin till it became non adhesive, sheets of stratum corneum were removed onto pieces, 1.5cm x 4.5cm, of an adhesive tape (Steri Drape) following a method described by Knight (1972).
The tape is then mounted on a glass slide with its adhesive side uppermost and fixed at each end by small pieces of an ordinary adhesive tape. Strips were obtained from a volunteer from the following body areas, cheek, upper back, forearm, palm, abdomen, groin, leg and sole.

5:4:5 Growth assay:-

10ml volumes of arthrospore suspension (1 x 10^7/ml) were applied to and spread on the stripped stratum corneum. Strips were placed on a bent glass

rod in a sterile plastic petri dish (9cm x 9cm) containing 8ml of sterile distilled water and incubated at 37°C for varying lengths of time: 2, 4, 5, 6, 8, 12, 16, 24, 48 hr and 7 days. Strips were heat fixed at 45°C for 10-15 minutes and stained transversely with Periodic acid-Schiff (PAS) (Appendix). Strips were mounted in Von Apathy medium (Difco), covered with cover glasses (22mm x 50mm) and examined microscopically. Additionally, PAS stained strips of stratum corneum from the leg inoculated with arthrospores of T. mentagrophytes number 121 and incubated for 48 hr were counterstained with toluidine blue, sectioned transversely and examined microscopically. For control purposes arthrospores were inoculated onto pieces of Steri Drape without stratum corneum and incubated at 37°C for the various time intervals. They were prepared for light microscopy as described above.

5:4:6 Effect of temperature, humidity, oxygen and depth of stratum corneum on the germination of arthrospores:-

Sheets of stratum corneum from the leg were prepared and inoculated with 10ml of arthrospore suspension (1×10^7/ml) of T. mentagrophytes strain 121 as has been described above. Strips were incubated for 16 hr under the following conditions: 28°C, 4°C, 45°C, absence of moisture and anaerobically. To check the effect of the depth of stratum corneum, 10ml of the arthrospore suspension was applied directly to the leg, over a marked surface area of 1.5 x 4.5cm and subsequently the stratum corneum was stripped after the moisture had dried. Incubation was carried out as described previously at 37°C for 16 hr in a sterile plastic petri dish containing 8ml sterile distilled water.

5:4:7 Effect of exposing arthrospores in distilled water at 28°C for 24 hr on germination on stratum corneum:-

The method described in Chapter 4, Section 4:4:6 was used to treat arthrospores of the three strains of dermatophyte. Arthrospores were then resuspended in PBS and the concentration adjusted to 1×10^7/ml. Arthrospore germination was determined and then 10ml of arthrospore suspension was inoculated onto strips of stratum corneum from different body areas: sole, palm, cheek and forearm, employing the method described above. Strips of stratum corneum were incubated at 37°C for 6, 8 and 16 hr and then prepared for light microscopy as has been described above. Control incubation was carried out on pieces of Steri Drape without stratum corneum.

5:4:8 Electronmicroscopy of dermatophyte growth on stratum corneum:-

Strips of stratum corneum from the leg on pieces of Steri Drape were inoculated with arthrospores of T. mentagrophytes strain 121 and incubated at 37°C for the various time intervals as has been described above. Small pieces of the strips were cut and attached to 10mm aluminium stubs with colloidal silver paint. They were then sputter coated with gold and examined under a scanning electron microscope.

5:5:9 Electronmicroscopy of scale from human dermatophytosis:-

Scales from two human cases of tinea pedis were processed by standard methods for transmission electronmicroscopy.

5:4:10 Statistical analysis of results:-

The results were analysed by the Student's t-test.

5:5 Results

5:5:1 Viability of arthrospores:-

The viability of arthrospores as assessed by their germinative ability is shown in Table 5:1. An arthrospore was considered to have germinated when a visible germ tube had developed.

5:5:2 Growth of arthrospores on stripped sheets of stratum corneum.

By 4 hr incubation, arthrospore germination had started and with further incubation increased (Figure 5:1; Table 5:2). Cross sections of infected stratum corneum showed germ tubes growing transversely and through the thickness of the stripped stratum corneum (Figure 5:2). Control plates, i.e. arthrospores inoculated onto Steri Drape without stratum corneum, showed only occasional germination (Table 5:2) and the germ tubes were very short, thin and only faintly stained with PAS (Figure 5:3).

After 16 hr incubation it was not possible to quantitate arthrospore germination because of the high degree of growth and development of

Strains	Section 5:4:5 and 5:4:8	Section 5:4:6	Section 5:4:7
121	96.22±0.91	96.77±1.61	96.11±0.73
126	95.55±1.42	Not used	96.88±1.44
4	96.00±1.76	Not used	96.11±1.28

Table 5:1: - Viability of arthrospores. Data are the mean ± SD of germinated arthrospores and were calculated from three viability assays; each assay was read in triplicate. Data of each column relates to arthrospores used in the experiments of the section specified above the column.

Strain	Time	Sole	Leg	Groin	Abdomen	Back	Cheek	Forearm	Palm	Control
121	4 hours	3.11± 2.37	4.22± 5.24	0.33± 0.66	3.88± 1.72	8.11± 4.22	5.77± 4.82	2.44± 3.65	0.66± 1.33	0.00
	5 hours	23.77± 6.17	26.11± 8.69	13.77± 5.00	35.77± 8.82	36.55± 8.05	19.22± 2.89	24.22± 8.40	24.11± 8.42	0.00
	6 hours	34.77±11.39	44.22±10.06	28.11± 8.74	34.77± 6.39	41.66± 5.81	33.22± 4.18	34.77± 5.99	31.88± 7.86	0.00
	8 hours	49.33± 7.51	48.22± 9.46	44.22±16.85	45.33± 9.10	47.22± 9.16	42.22±7.28	42.33±13.17	34.11± 9.19	0.00
	12 hours	59.44± 6.14	61.44± 7.35	63.88± 9.74	56.44±11.47	73.33±11.70	50.44±10.95	53.63±10.72	46.77± 8.72	0.00
	16 hours	79.11± 8.29	75.11±10.50	79.22± 9.77	84.77± 5.69	85.77± 4.21	77.00±11.84	76.00± 7.05	71.88± 6.02	1.22±1.13
4	6 hours	0.77± 0.91	1.44± 1.70	2,22± 3,35	3.44± 2.58	4.55± 3.49	2.77± 1.93	3.22± 3.22	2.66± 3.74	0.00
	8 hours	6.66± 3.82	4.11± 1.52	6.00± 4.13	8.11± 2.76	13.33± 4.54	7.55± 4.59	6.88± 3.54	13.88± 3.69	0.00
	16 hours	65.77± 3.88	55.33± 4.94	55.44± 4.16	57.55± 2.26	61.44± 5.96	62.00± 7.07	57.55± 6.14	58.00± 5.83	0.00
126	6 hours	8.44± 5.29	6.66± 6.18	3.33± 1.94	9.11± 2.55	10.44±3.68	15.88± 4.01	9.66± 3.09	9.11± 5.21	0.00
	8 hours	20.33± 7.74	17.55± 5.88	19.44± 6.92	21.33± 4.34	18.77± 6.59	26.55± 7.31	20.33± 9.27	21.77± 6.86	0.00
	16 hours	59.00± 6.23	53.77± 3.52	56,33± 7.07	56.66± 4.89	62.44± 6.11	57.55± 4.74	54.22± 9.87	55.55± 5.07	3.44±1.06

Table 5:2: - Germination of arthrospores on stratum corneum from various body areas. Data are the mean ± SD of percentages of germinated arthrospores and were calculated from three assays; each was read in triplicate.

Figure 5:1a

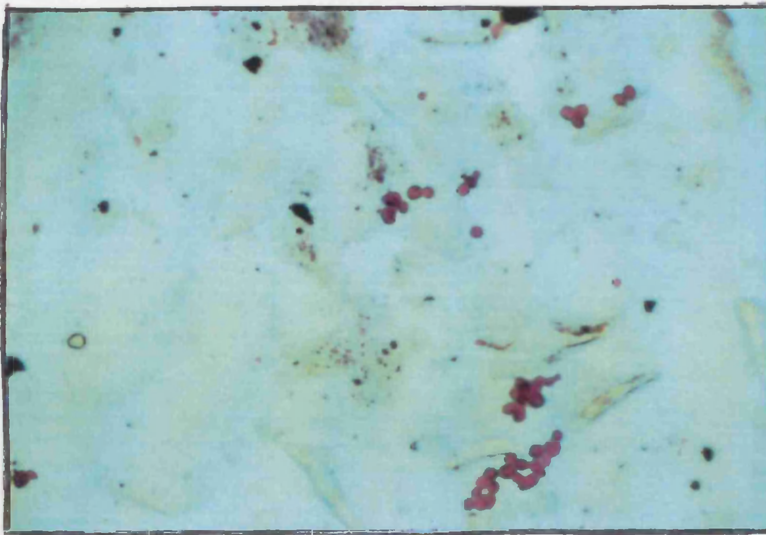


Figure 5:1b

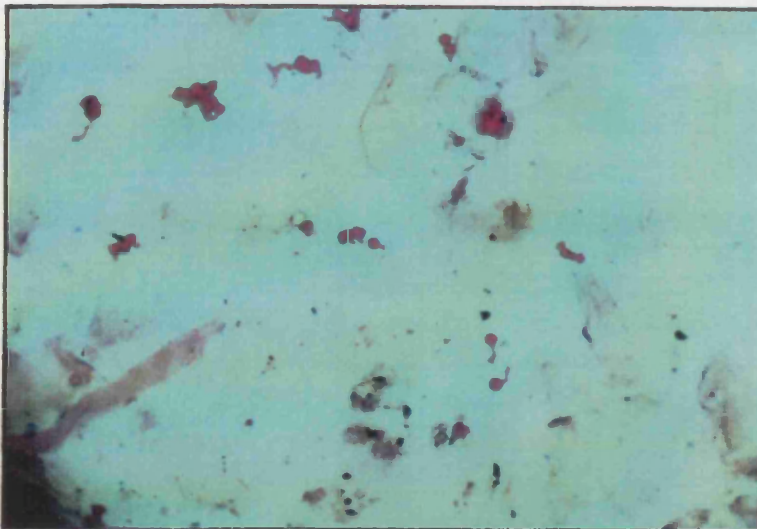
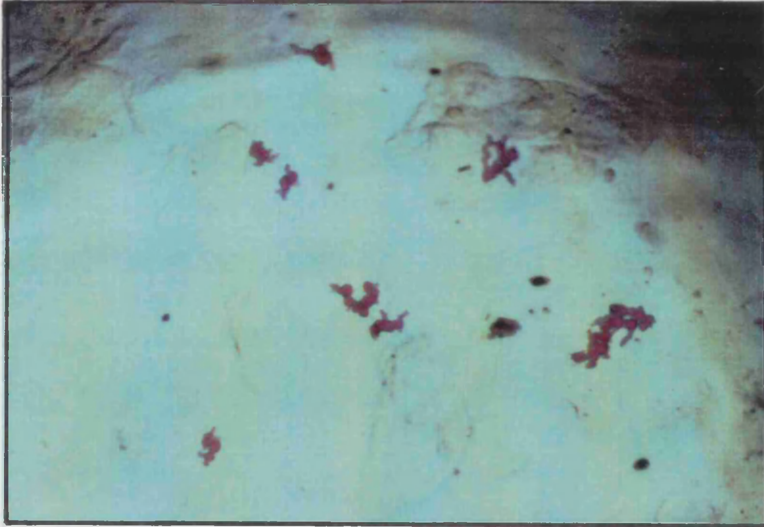


Figure 5:1c



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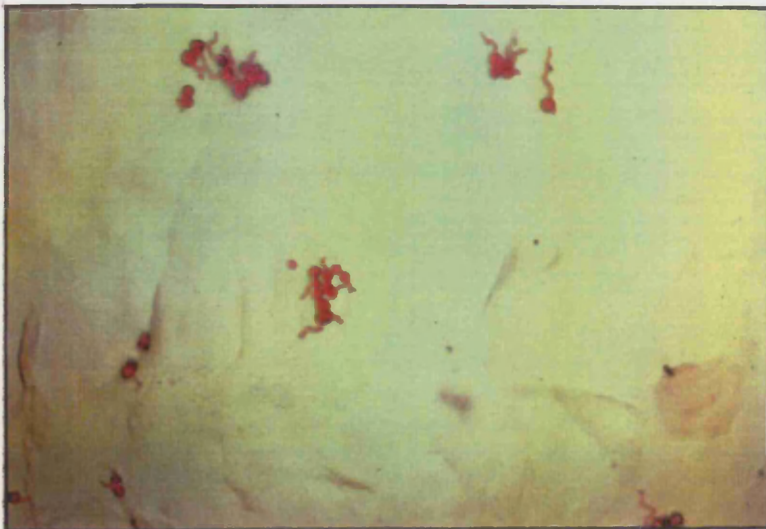

Figure 5:1e

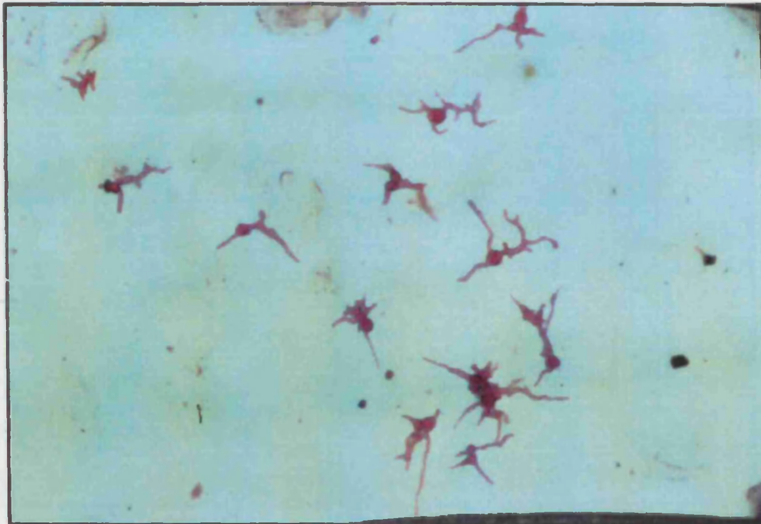

Figure 5:1:- Arthrospores inoculated on stratum corneum and incubated at 37°C for 4 hr (a), 6 hr (b), 8 hr (c), 12 hr (d) and 16 hr (e). By 4 hr incubation, germination had started and increased with time up to 16 hr when long branching germ tubes had developed, P.A.S. stain, a X 378, b - e X 252.

Figure 5:2a

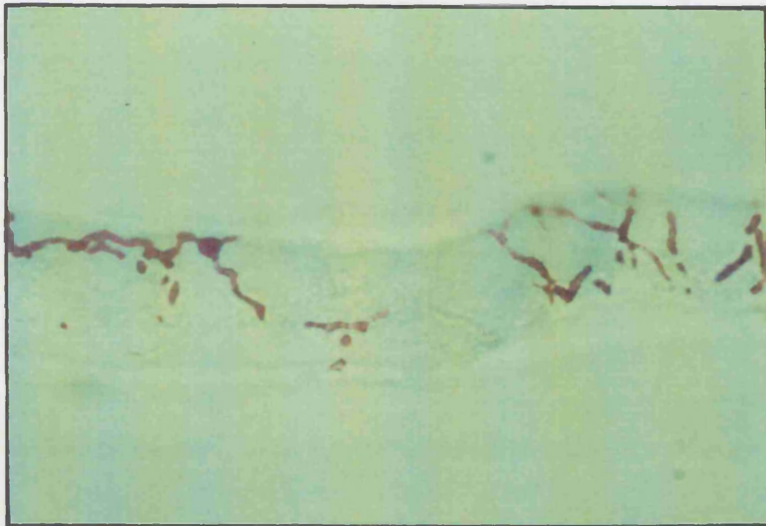

Figure 5:2b

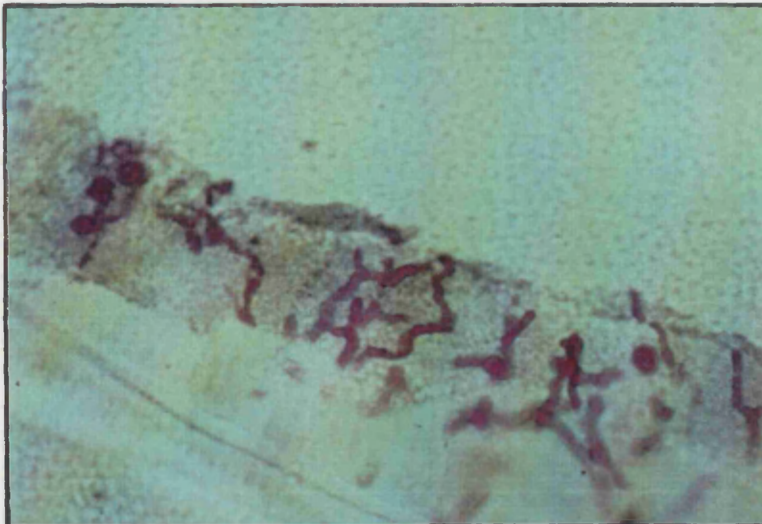

Figure 5:2:- Cross-section of arthrospore-inoculated stratum corneum and incubated at 37°C for 48 hr (a and b). Germ tubes penetrated transversely and through the thickness of the stratum corneum, P.A.S. stain and toluidine blue counterstain, X 630.

Figure 5:3a

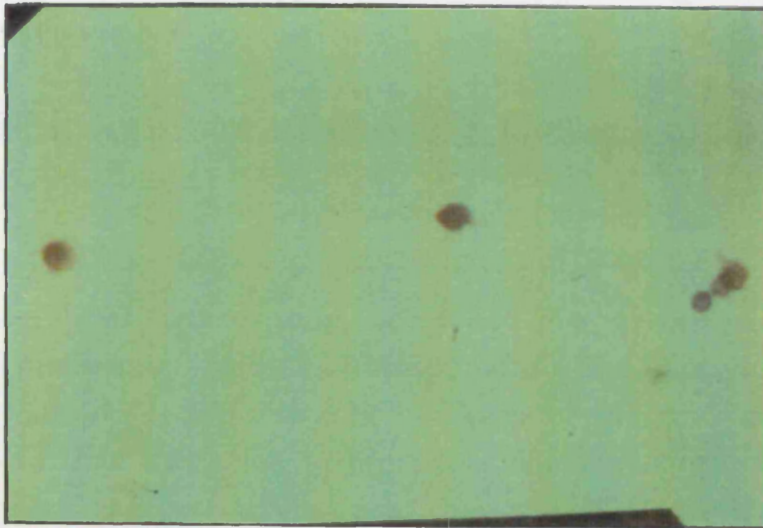

Figure 5:3b

Figure 5:3:- Arthrospores inoculated on Steri Drape without stratum corneum and incubated for 16 hr (a) and 7 days (b). Very low level of germination occurred and germ tubes were short, thin and faintly stained with P.A.S., a X 787, b X 630.

vegetative mycelia (Figure 5:4). At 16 hr statistically significant differences in germination were found between strains 121 and 126; and 121 and 4 in stratum corneum from all the tested sites, P values were less than 0.01.
By 7 days incubation, masses of branched hyphae had broken down into arthrospores but some hyphal branches and terminal segments remained aseptate (Figure 5:5).

5:5:3 Effect of exposing arthrospores in distilled water at 28°C for 24 hr on germination:-

At the end of the treatment period in distilled water at 28°C for 24 hr no germination of arthrospores occurred. The germination on sheets of stratum corneum of exposed arthrospores at 6 and 8 hr with the exception of those of strain 121 and 4 at 6 hr on sheets of stratum corneum from palm, was more than untreated arthrospores and in some instances reached a statistically significant difference, as shown in Tables 5:3 and 5:4.

5:5:4 Effect of temperature, moisture, oxygen, and depth of layer of stratum corneum on germination of arthrospores:-

No germination of arthrospores occurred after 16 hr incubation of the infected strips of stratum corneum in the absence of moisture or oxygen, at 45°C or at 4°C (Table 5:5). Germination of arthrospores at 28°C or over the external surface of the stratum corneum was comparable to that at 37°C over the stripped stratum corneum from the corresponding body area (leg).

5:5:5 Ultrastructure of arthrospore growth on stratum corneum:-

Scanning electron microscopy showed that more than one layer of the stratum corneum was stripped (Figure 5:6). Emerged germ tubes could be seen either appossed over the surfaces of corneocytes, insinuated between corneocytes or attached to the margin of corneocytes (Figure 5:7). Small germ tubes were also seen with their tips which appeared to be pointing towards the corneocytes (Figure 5:8). Long germ tubes either extended over corneocytes or appeared to be buried producing ridges (Figure 5:9). Points of entry of germ tubes into stratum corneum and into corneocytes, where bulges developed, were also observed (Figure 5:10). Arthrospore formation was seen in samples after 7 days incubation but some hyphal branches and terminal segments remained aseptate (Figure 5:11). In infected samples after

Figure 5:4a

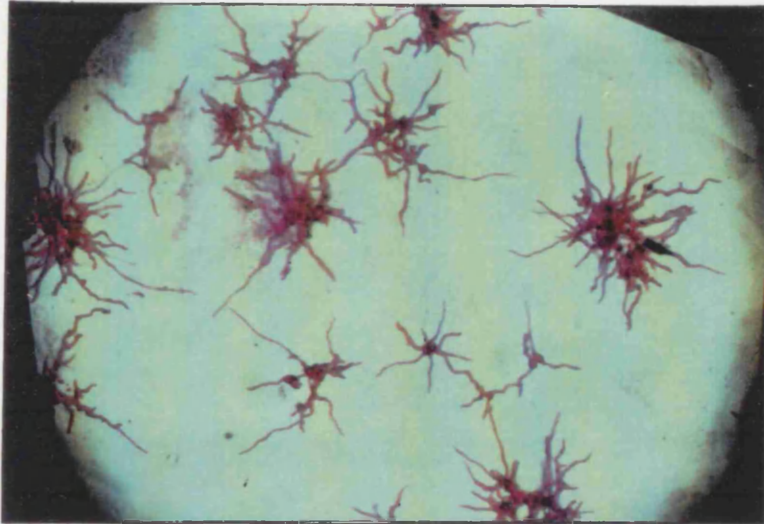

Figure 5:4b

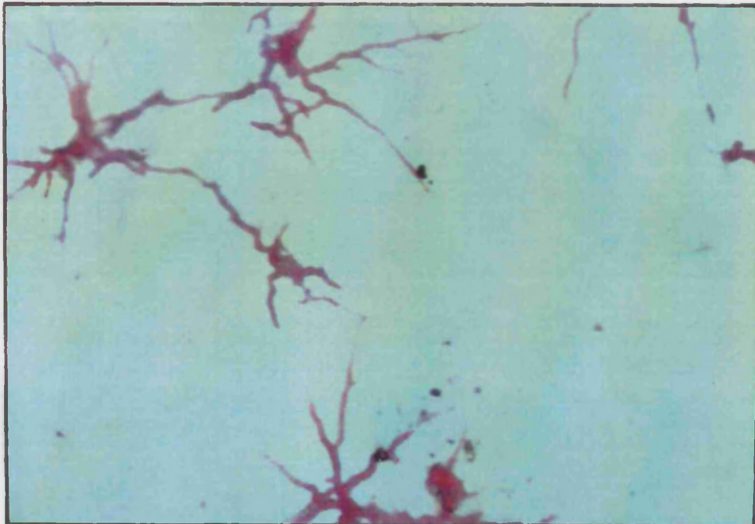

Figure 5:4:- Arthrospores inoculated on stratum corneum and incubated at 37°C for 24 hr (a) and 48 hr (b). There is a distinct formation of fungal microcolonies when arthrospores had multigerminated and germ tubes multibranched and extended across corneocytes, P.A.S. stain, a X 252, b X 378.

Figure 5:5a

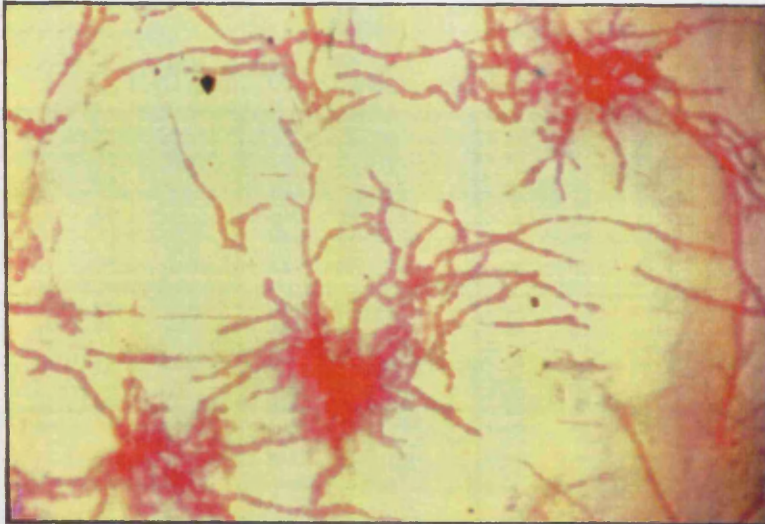

Figure 5:5b

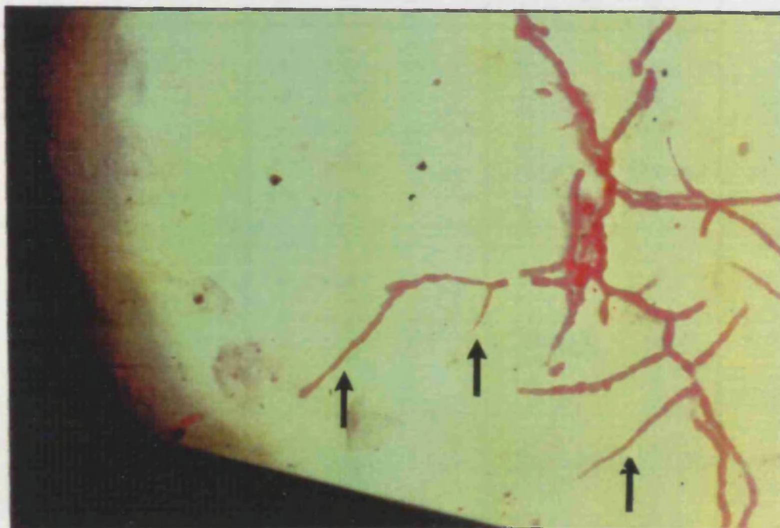

Figure 5:5:- Arthrospores inoculated on stratum corneum and incubated at 37°C for 7 days (a and b). Masses of branched hyphae have broken down into arthrospores but some segments of hyphae have remained aseptate (arrows in b), P.A.S. stain, a X 252, b X 315.

Strain	Time	Sole	Palm	Cheek	Forearm	Control
121	6 hours	44.66± 8.51	30.66±6.32	37.77± 7.26	35.00±10.61	3.44±0.49
	8 hours	59.77± 3.67	48.55±10.89	50.00±12.66	47.00±12.92	6.88±0.87
	16 hours	72.88±10.82	71.44± 4.94	65.55± 9.33	69.66± 7.71	6.66±1.33
4	6 hours	9.22± 5.49	1.88± 1.91	8.44± 2.87	7.22± 4.46	0.00
	8 hours	22.66± 5.61	24.22± 8.91	26.77± 6.19	30.77± 8.20	0.00
	16 hours	65.33± 2.90	55.66± 5.22	61.66± 5.24	64.00± 6.58	0.00
126	6 hours	39.00±11.10	24.66± 9.14	30.66±14.40	33.33±15.93	4.44±1.83
	8 hours	46.88±10.09	39.88±11.11	42.11±12.77	44.44±11.91	6.33±1.82
	16 hours	60.33± 6.53	56.44± 4.52	65.66± 7.80	62.88± 7.57	7.00±1.69

Table 5:3: - Effect of exposing arthrospores in distilled water at 28°C for 24 hr on germination on stratum corneum from various body areas. Data are the mean ± SD of percentages ofgerminated arthrospores and were calculated from three assays; each was read in triplicate.

Strain	Time	Sole	Palm	Cheek	Forearm
121	6 hours	>0.1	>0.5	<0.05	>0.5
	8 hours	<0.01	<<0.001	>0.05	>0.1
	16 hours	>0.1	>0.5	>0.5	>0.05
4	6 hours	<0.01	>0.5	<<0.001	<0.01
	8 hours	<<0.001	<0.02	<<0.001	<<0.001
	16 hours	>0.5	>0.5	>0.5	>0.05
126	6 hours	<<0.001	<0.01	<0.05	<0.01
	8 hours	<<0.001	<<0.001	<0.02	<<0.001
	16 hours	>0.5	>0.5	>0.05	<0.05

Table 5:4: - P values of differences between germination of arthrospores exposed in distilled water at 28°C for 24 hr and unexposed (freshly harvested) arthrospores.

<< = P value is exceedingly less than 0.001

Factor	germination
Desiccation	0.00
Anaerobiasis	0.00
45°C	0.00
4°C	0.00
28±C	76.66±4.89
depth of stratum corneum	79.88±4.62

Table 5:5: - Germination of arthrospores under various environmental conditions. Data are the mean ± SD of percentages of germinated arthrospores and were calculated from three assays; each assay was read in triplicate.

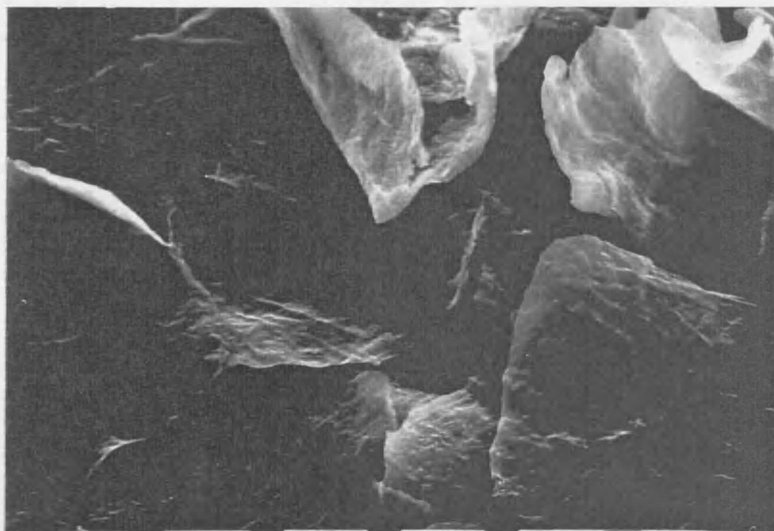

Figure 5:6:- Scanning electronmicrograph of stratum corneum stripped on Steri Drape. More than one layer are present on the tape, X 1740.

Figure 5:7a

Figure 5:7b

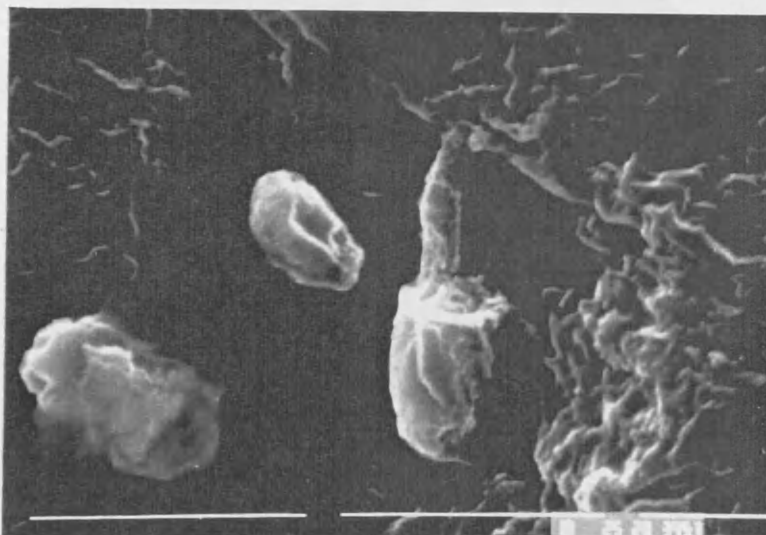

Figure 5:7c

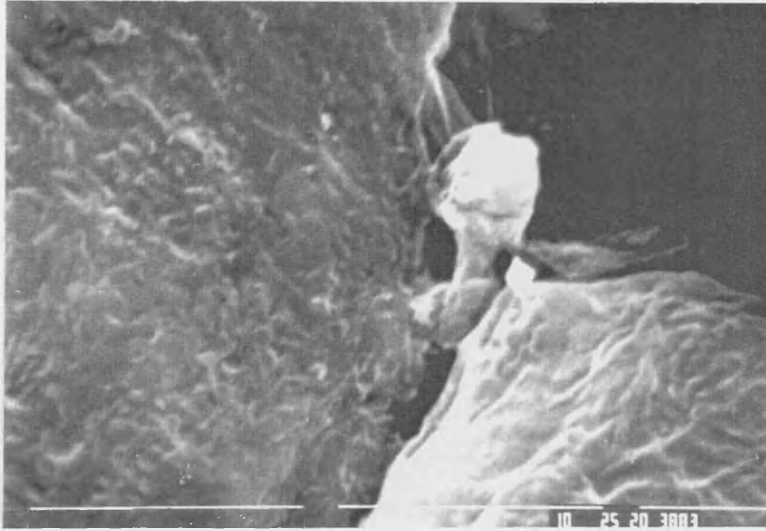

Figure 5:7:- Scanning electronmicrographs of arthrospores inoculated on stratum corneum and incubated at 37°C for 6 hr (a, b and c). Germ tubes are seen either appressed to the surface of corneocytes (a), attached to the margin of corneocyte (b), or insinuated between corneocytes (c), a X 1740, b and c X 5800.

Figure 5:8a

Figure 5:8b

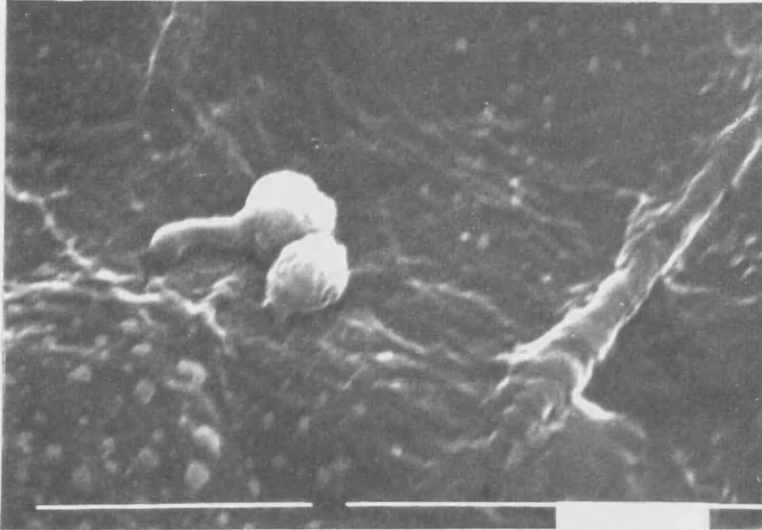

Figure 5:8c

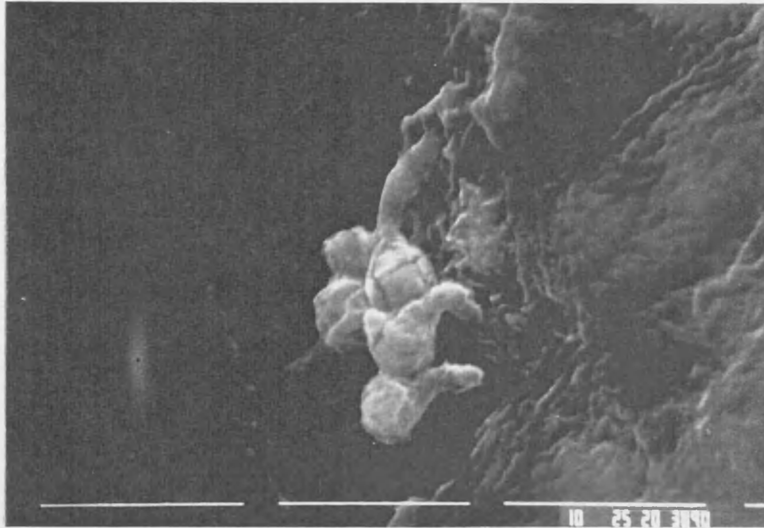

Figure 5:8:- Scanning electronmicrographs of arthrospores inoculated on stratum corneum and incubated at 37°C for 6 hr (a,b and c). Tips of germ tubes appear to be inserted into corneocytes (a,b and c), X 4060.

Figure 5:9a

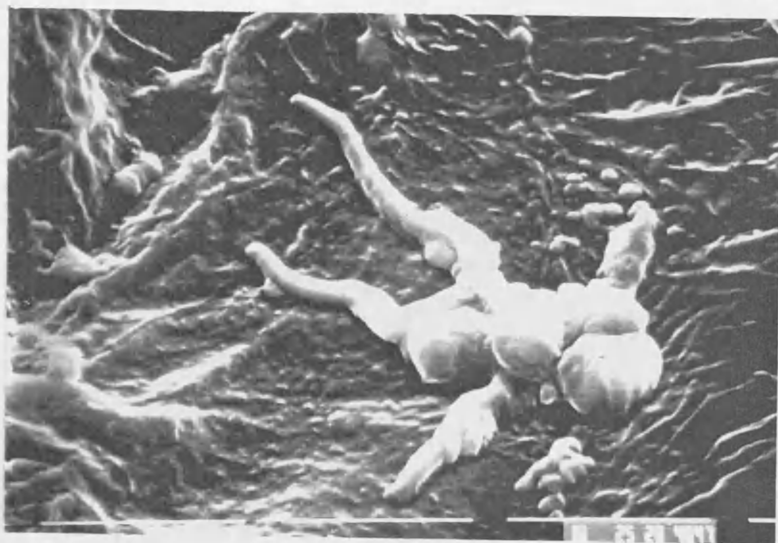

Figure 5:9b

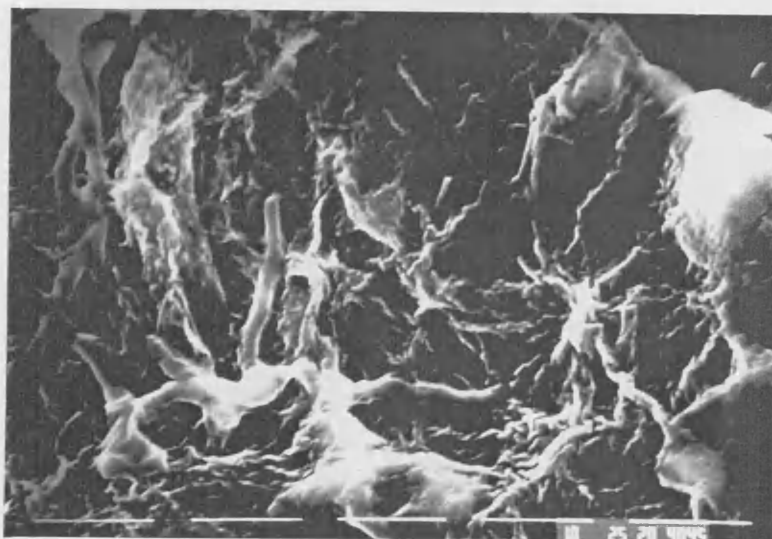

Figure 5:9c

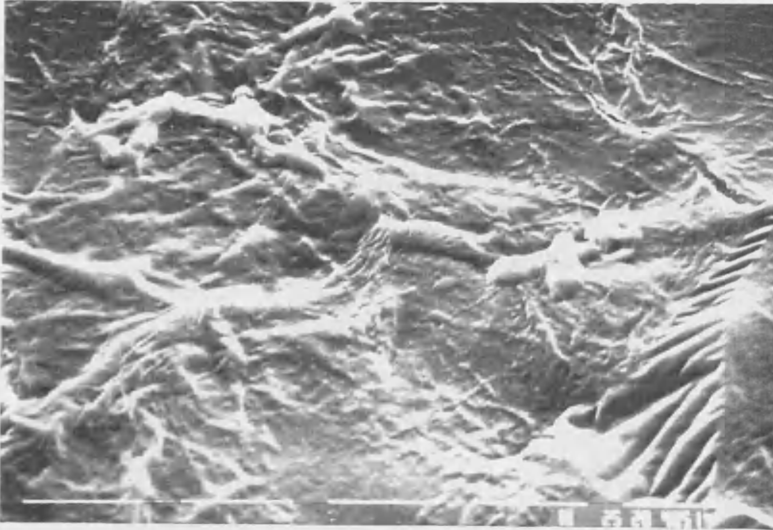

Figure 5:9d

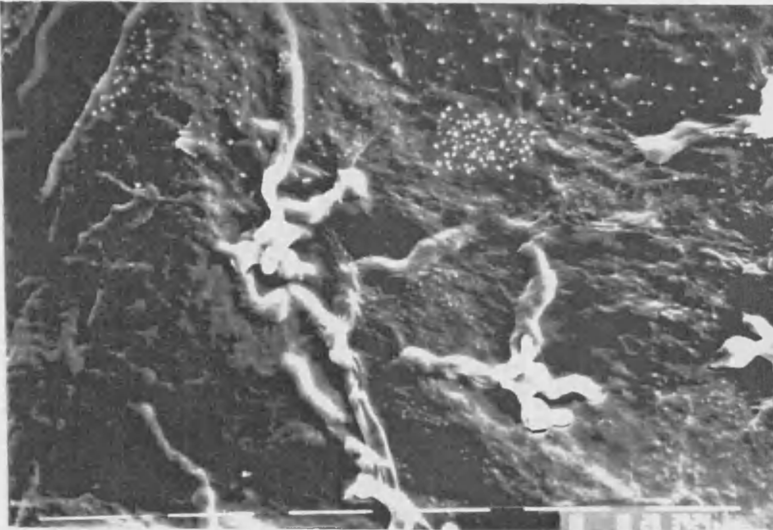

Figure 5:9:- Scanning electronmicrographs of arthrospores inoculated on stratum corneum and incubated at 37ºC for 16 hr(a,b and c) and 48 hr (d). Germ tubes are seen extending over the surface of corneocytes (a and b) or appear to be buried in them producing ridges (c and b), a. b and c x 2320, d x 1740.

Figure 5:10a

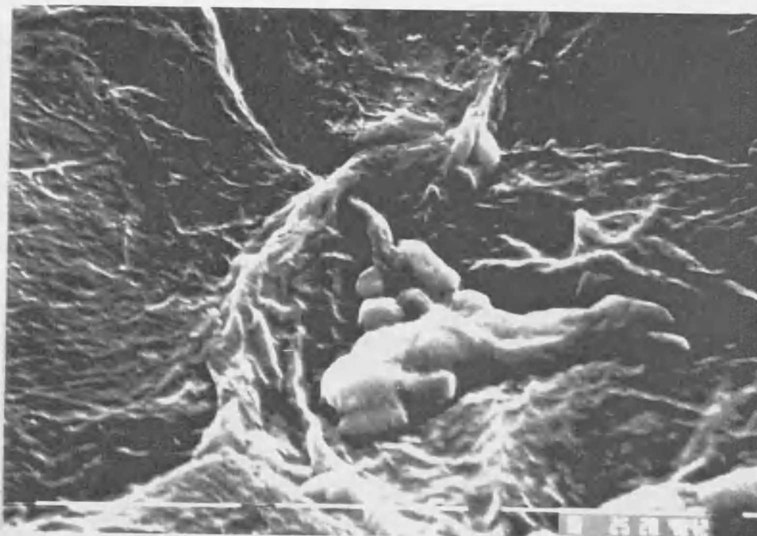

Figure 5:10b

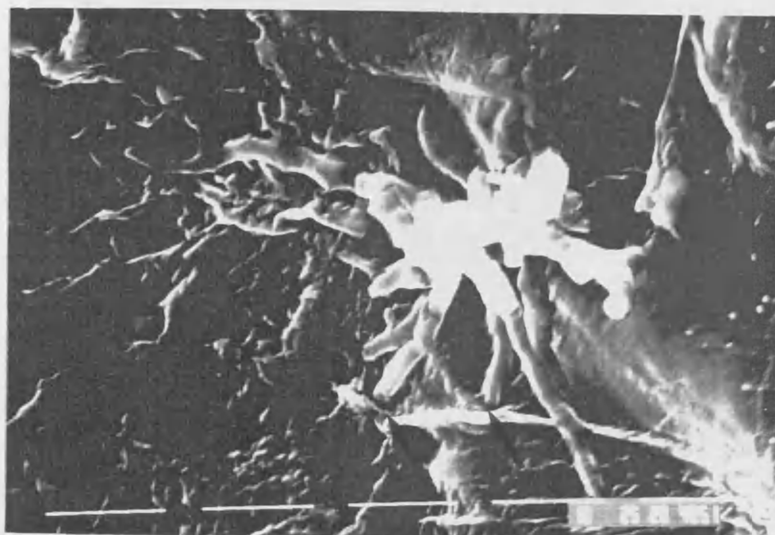

Figure 5:10c

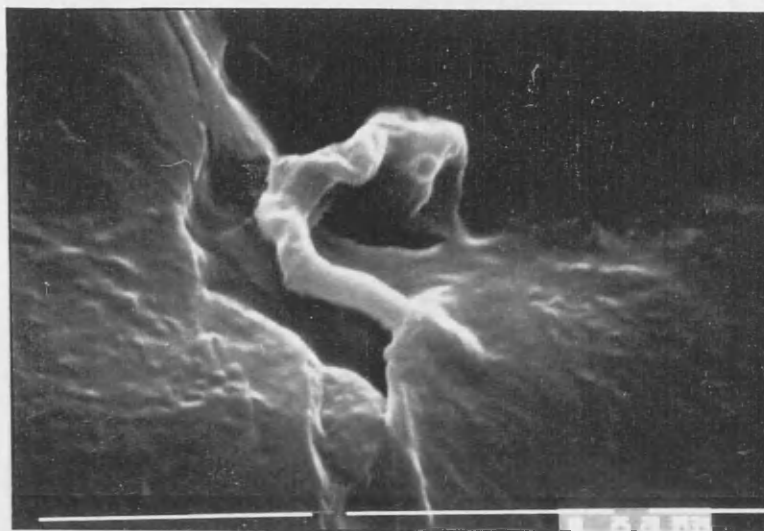

Figure 5:10:- Scanning electronmicrographs of arthrospores inoculated on stratum corneum and incubated at 37°C for 16 hr (a,b and c). Points of penetration of germ tubes into the stratum corneum (arrows in a and b) and into corneocyte where bulge had appeared (c) are seen, a and b x 2320, c X 5800.

Figure 5:11a

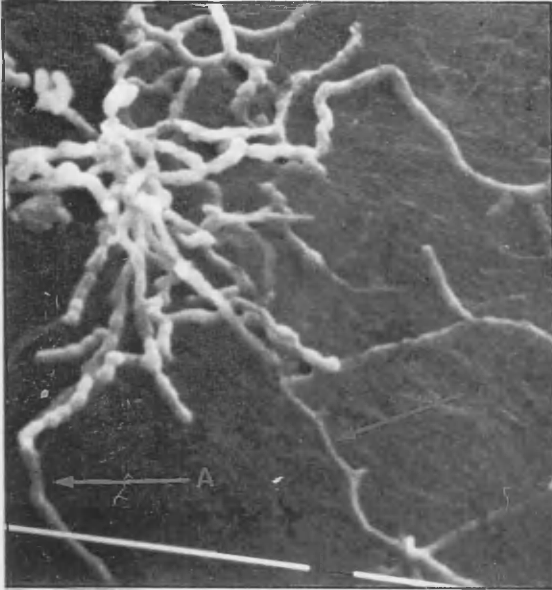

Figure 5:11b

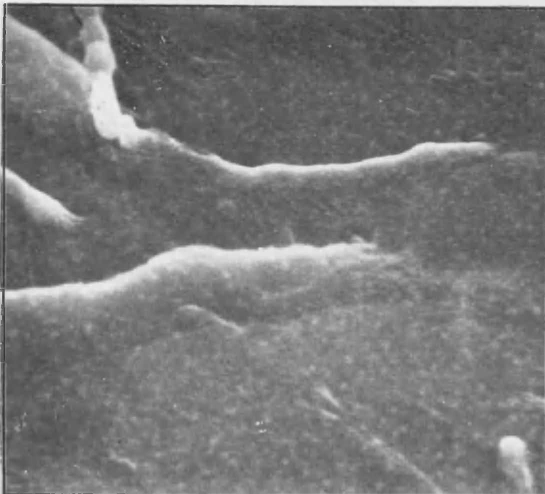

Figure 5:11:- Scanning electronmicrographs of arthrospores inoculated on stratum corneum and incubated for 7 days (a and b). The corneocytes appear disintegrated and hyphae have broken down into arthrospores but with some segments of hyphae remaining unfragmented (arrows). Figure 5:11 b is a higher magnification of a hyphal segment (arrow a) in Figure 5:11 a, a X 1500, B X 2490.

7 days incubation the corneocytes appeared damaged (Figure 5:11), unlike those which were incubated without arthrospores where the corneocytes appeared unchanged (Figure 5:12). Arthrospores which were lying on bare areas of the tape were noticed to germinate too (Figure 5:13).

5:5:6 Ultrastructure of corneocyte-dermatophyte relationship in scales from lesions of dermatophytosis:-

Transmission electronmicroscopy was used to examine scales from two cases of human tinea pedis. Fungal elements were seen located within the keratin (Figure 5:14). In one case the fungal elements had thick walls. In both cases electron transparent zones were seen around the fungal elements.

5:6 Discussion:-

The results which have been obtained from this study can, in terms of growth of arthrospores on stratum corneum, be arranged in the following sequence: germination of arthrospores, penetration of stratum corneum and arthrospore formation.

5:6:1 Germination of arthrospores on stratum corneum:-

After 2 hr incubation no germination of arthrospores has been noticed but the appearance of arthrospores on the stripped stratum corneum (Figure 5:15) may create an impression of an in vivo situation where arthrospores adhere to corneocytes. By 4 hr incubation arthrospore germination had started and increased with time. The reason for this time-dependent increase in germination is not known fully but as has been discussed in Chapter 4, Sections 4:2 and 4:6:1, may be related to the morphological and physiological changes which occur in spores in general during the germination process. Treating the arthrospores in distilled water at 28°C for 24 hr prior to their inoculation onto the stratum corneum resulted in an increase in germination. This increase was at occasions of 6 and 8 hr incubation of statistically significant levels as shown in Tables 5:3 and 5:4. The epidemiological significance of this increase in germination of arthrospores after treatment in distilled water has been discussed in Chapter 4, Section 4:6:2.

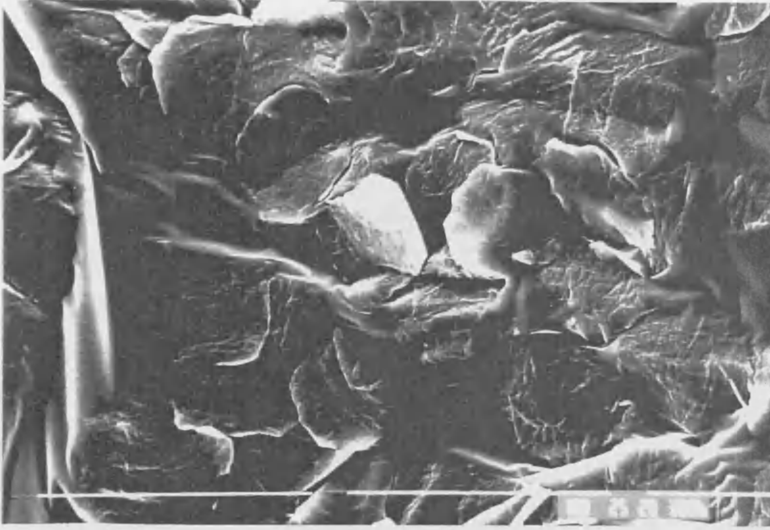

Figure 5:12:- Scanning electronmicrograph of control stratum corneum, not inoculated with arthrospores, after incubation at 37°C for 7 days. The corneocytes appear unchanged, X 750.

Figure 5:13a

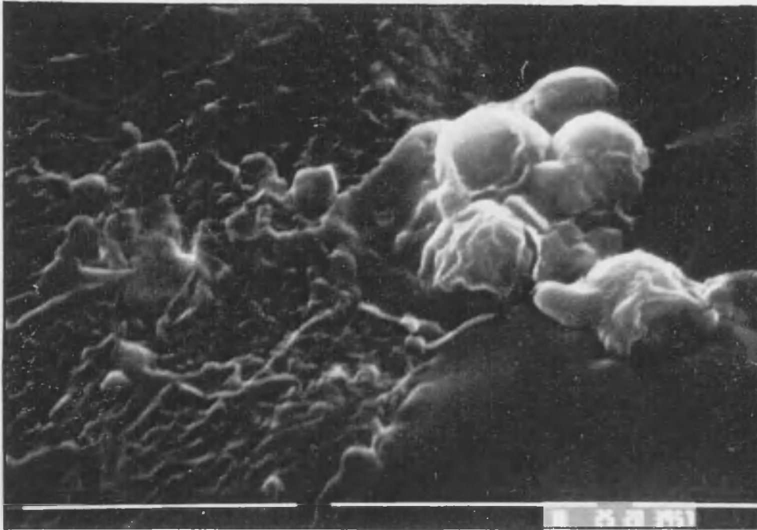

Figure 5:13b

Figure 5:13:- Scanning electronmicrograph of stratum corneum inoculated with arthrospores and incubated at 37°C for 6 hr (a and b). Arthrospores lying on bare areas of tapes are seen to have germinated, a X 8300, b X 8750.

Figure 5:14a

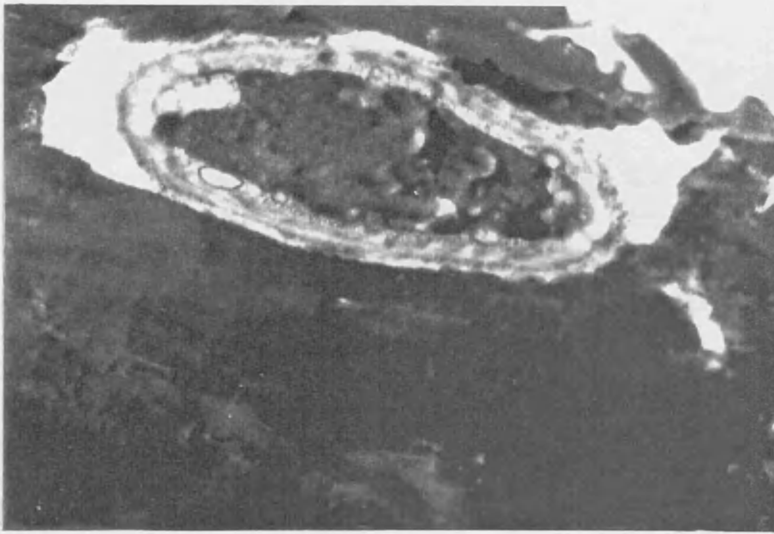

Figure 5:14b

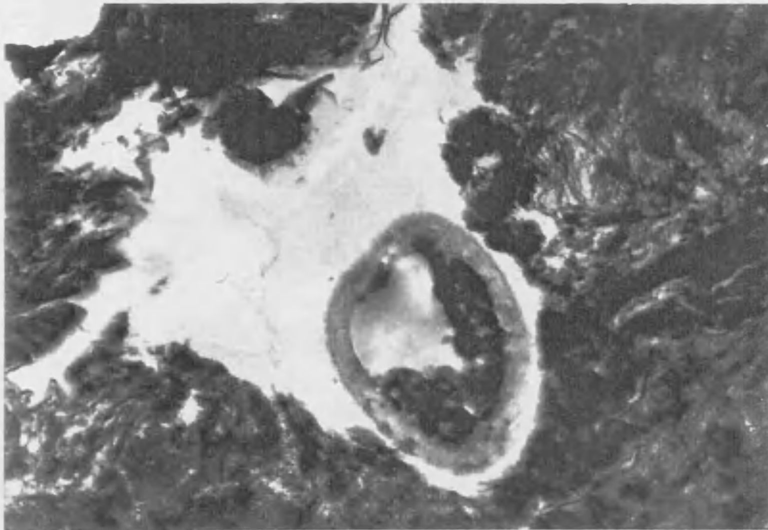

__Figure 5:14c__

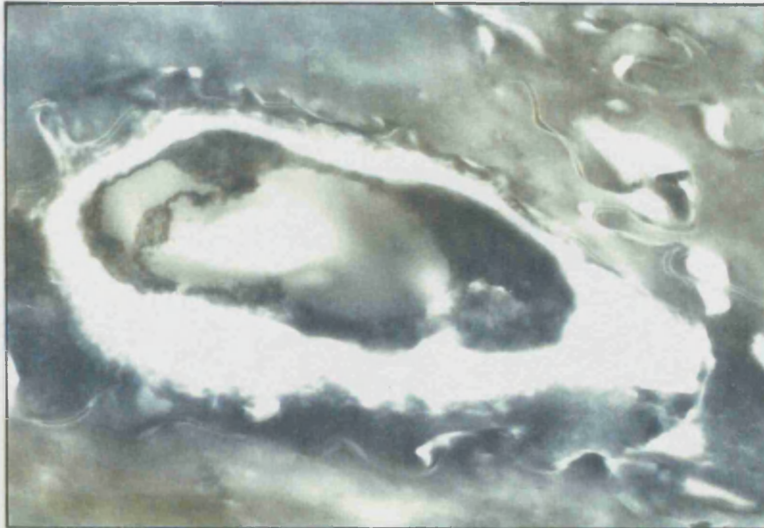

__Figure 5:14:-__ Transmission electronmicrographs of scales from human cases of tinea pedis. Fungal elements are seen situated within the keratin, a X 17400, b X 20250, c X 26700.

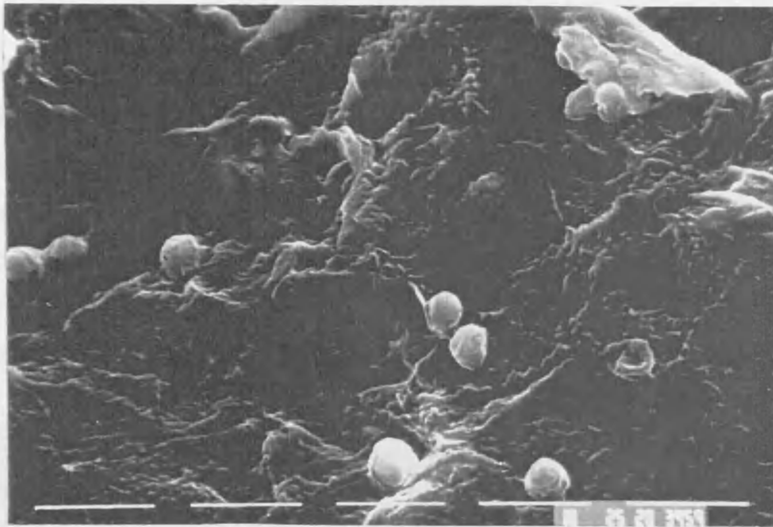

Figure 5:15:- Scanning electron photomicrograph of arthrospores inoculated on stratum corneum and incubated at 37°C for 2 hr. By this time arthrospore germination has not been observed but the appearance of arthrospores on the stratum corneum may create impression of the in vivo situation of arthrospore adherence to corneocytes, X 4000.

Germination of arthrospores on stratum corneum from areas rich in sebaceous glands, e.g. cheek and upper back was comparable to that on stratum corneum from areas devoid of them, i.e. palms and soles (Table 5:2). This may indicate that, germination of arthrospores is less likely to be affected by sebum. This is unlike the spontaneous clearance of scalp dermatophytosis at puberty which has been connected to a change in sebum at that age (Rothman and Lorincz, 1963) and may be related to the reported inhibitory action of synthetic sebum on the mycelial growth of dermatophytes (Yousef et al ., 1978). Alternatively, it might be that the amount of sebum in the stripped stratum corneum was not enough to interfere with the germination process. However, it has been reported that arthrospores are more resistant than their hyphal counterparts to common antimycotics such as clotrimazole, griseofulvin and miconazole (Hashimoto and Blumenthal, 1978) and the germination process is more resistant to imidazole antifungals than is germ tube extension (Scott et al ., 1984).

In the previous study, (Chapter 4), it was found that adherence to corneocytes was not a necessary prelude to arthrospore germination. In the present study this was confirmed by observing arthrospores which were lying on bare areas of the tape producing germ tubes (Figure 5:13). This finding, in addition to the observation that arthrospores had germinated while they were located extracellularly, suggests that a germination promoting factor(s) either was present outside the corneocytes or has diffused out from them or both. This point has been discussed further in Chapter 4, Section 4:6:1.

Table 5:5 shows the effect of certain experimental factors on the germination. Germination of arthrospores was found to be oxygen dependent and did not occur in the absence of moisture, at 4°c and at 45°c. At 28°C arthrospore germination was not statistically different from that at 37°C. The pathogenic significance of these observations is their similarity to the natural conditions found on the skin (Chapter 1, Section 1:5). The germination of arthrospores applied directly to the skin, i.e. to the outermost layer of stratum corneum, prior to stripping stratum corneum was not different from that obtained in the ordinary method where arthrospores were inoculated onto an inner layer of stratum corneum since stripping resulted in making the outermost layer lowermost and an inner layer uppermost on the tape. This shows the ability of arthrospores to germinate on the surfaces of corneocytes from different levels of stratum corneum. This observation is, however, limited to the upper two thirds of the stratum corneum because repeated stripping of the stratum corneum

with adhesive tape was reported to remove only two thirds of the thickness of this tissue (Hojyo Tomoka and Kligman, 1972).
In control tapes, i.e. without strips of stratum corneum, germination of arthrospores was of a very low level (Table 5:2) and germ tubes were short, thin and faintly stained with PAS (Figure 5:5). This is in contrast to the multibranched, long and deeply stained germ tubes which developed on the stratum corneum after the same period of incubation (Figure 5:7 and 5:8). This probably reflects the ability of germ tubes to utilize components of stratum corneum (Chapter 1, Section 1:5) as growth substrate.
Statistically significant differences, P values were less than 0.01, were found between the germination of T. mentagrophytes number 121 and both T. mentagrophytes number 126 and T. interdigitale number 4 at 16 hr incubation. These differences support the suggestion mentioned in Chapter 4, Section 4:6:2 that germination of arthrospores should be regarded as a pathogenic factor in dermatophytosis.

5:6:2 Penetration of stratum corneum by germ tubes:-

The penetration of stratum corneum started by the emergence of germ tubes from the arthrospores which were lying extracellularly. Germ tubes were seen either appressed to, insinuated in between or attached to the margin of corneocytes (Figure 5:6). These arrangements of germ tubes, in addition to the ability of arthrospores to adhere to corneocytes, are likely to secure the whole fungal structure in position over the stratum corneum. With further development, germ tubes extended horizontally in and through the thickness of the stratum corneum (Figure 5:2 and 5:3). The horizontal extension is likely to result in the clinically observable sign of peripheral expansion of lesions of dermatophytosis. Knudsen (1975) cultured dermatophytes from skin around the margin of lesions for a distance up to 6cm. The penetration of germ tubes to deeper layers of the stratum corneum has also been reported in experimentally induced dermatophytosis in guinea pigs and found to occur by a zoophilic T. mentagrophytes to an extent greater than that seen by anthropophilic T. mentagrophytes (Fujita and Matsuyama, 1987). In vivo , growth of dermatophytes horizontally and through the thickness of stratum corneum is counteracted by the epidermal proliferative and exfoliative processes (Chapter 1, Section 1:5). Thus to maintain their hold on the stratum corneum dermatophytes should at least keep pace with these two processes of the epidermis.
Penetration of stratum corneum by the elongating germ tubes is associated with invasion of individual corneocytes (Figure 5:10). This was also evident

in the study of arthrospore germination in suspensions of corneocytes (Chapter 4). In candidosis a similar mode of mucosal cell penetration has been reported (Montes and Wilborn, 1985). The membranes of corneocytes are resistant structures (Blank, 1987). The mechanism by which germ tube rupture the corneocyte membrane (Figure 5:10) is not clear, but dermatophytes are known to be equipped with a variety of proteo and lipolytic enzymes (Chapter 1, Section1:5) including phospholipases (Das and Banerjee, 1977). Phospholipase activity was reported in C. albicans and was thought to play a part, by damaging the cell membranes, in the invasion of host tissue by hyphae. (Pugh and Cawson, 1975). However, the relevance of dermatophyte phospholipase activity to the invasion of corneocytes is still unknown. Alternatively, being filamentous structures, the dermatophytes may resort to mechanical forces to rupture the membranes of corneocytes. From the aforementioned discussion, it can be concluded that the elongation of germ tubes is a pathogenic factor in dermatophytosis.

The intracellular location of dermatophytes has also been observed in the scales from naturally occurring human infections (Figure 5:14). It was also reported in skin specimens from patients with tinea pedis and tinea cruris (Miyazaki, Seiji and Takaki, 1966) as well as from experimentally induced dermatophytosis in guinea pigs (Hetherington, Freeman and Halloran, 1969).

The clear zones seen around the fungal elements in the scales in Figures 5:14 possibly have resulted from a proteolytic activity of dermatophytes. Digestion of keratin in experimentally induced lesions in animals has also been suggested (Poulain et al., 1974) and keratinases have been detected in lesions of experimental dermatophytosis in guinea pigs using fluorescent antibody tests (Collins et al., 1973) and of plantar skin in human tinea pedis by immunoelectron microscopy (Koga et al., 1986). On the other hand, mechanical keratolysis, i.e. disintegration of keratin following invasion of non fibrillary matrix has been suggested as the means by which dermatophytes destroy hair and nail (Raubitschek, 1961). This suggestion was based on the finding of growth of dermatophytes on aqueous extracts from hair and nail. A finding similar to this was discussed previously in this study, i.e. the germination of arthrospores outside corneocytes and even on bare areas of the Steri Drape tape (Figure 5:13). The probable conclusion from Raubitschek's (1961) and the present study is therefore that the non-keratinous material of the cornified tissues can also support growth of dermatophytes. Additionally, studies with the electron microscope of infected hair (Tosti et al., 1970) have shown that dermatophytes may initially invade the hair by dissolving non-keratinous material therefore

disassembling the structure of hair but in later stages of invasion, keratin fibrils break down and even disappear forming ghost cells. Tosti et al . (1970) concluded that such complete destruction of keratin could not be mechanical and suggested that the dermatophytes can destroy keratin fibrils eventually. Similarly in nail infections, the presence of tunnels, thought to be the result of disintegration of the keratin has been reported (Alkiewicz and Sowinski, 1967). In the present study corneocytes incubated in the presence of the dermatophytes for 7 days appeared damaged as if disintegrated (Figure 5:11) unlike the control samples of corneocytes (Figure 5:12).

5:6:3 Formation of arthrospores on stratum corneum:-

By 7 days incubation some of the hyphae broke down into arthrospores (Figure 5:4 and 5:11) putting an end to the vegetative growth and completing their life cycle. An arthrospore therefore can give rise to more than one arthrospore depending on the degree of mycelial branching. The factor which has conditioned arthrospore formation in this study is probably the temperature of incubation i.e. 37°C. This temperature has been found to induce arthrospore formation in dermatophytes (Chapter 2). The presence of some hyphal branches or terminal segments which have remained aseptate suggests that these branches and terminal segments may continue to grow while others form arthrospores. Septate and aseptate hyphae have been observed in the same culture (Chapter 2, Section 2:6:6) and reported by others along with the presence of groups of arthrospores in a large amount of material from lesions caused by various dermatophytes (Onsberg, 1979). Specifically it was reported (Hashimoto et al., 1984) that complete cessation of hyphal tip elongation was not required for the initiation or even progression of arthrosporogenesis when it occurred in terminal hyphal segments and arthrospore formation could begin in any segment of a hypha regardless of its position (terminal, subterminal and intercalary) or its length. Also in hair infection it was found that, as hyphae proceed downward into the hair shaft they leave behind chains of arthrospores (Tosti et al., 1970). The combination of growth (hyphal elongation) and arthrospore formation is likely to help the dermatophytes to escape the epidermal exfoliative process while at the same time producing infective particles.
The stratum corneum is composed of several layers of tightly packed corneocytes with a narrow well-filled intercellular space (Blank, 1987; Chapter 1, Section 1:4). The dermatophytes grow horizontally and through

the thickness of the stratum corneum. Thus, from the structural point of view of the stratum corneum, arthrospores, because they are produced simply by fragmentation of hyphae, are the most suitable of the dermatophyte spores for the growth of these fungi in the stratum corneum, unlike micro and macroconidia which require a space for their lateral or apical development on hyphae. The site of stratum corneum was found to influence the diameter of hyphae; Gotz (1959) reported that, in the stratum corneum of the palms and soles which had a thickness of about 600 µm, hyphae of T. rubrum developed a diameter between 1.5µm - 2.5µm, while in the stratum corneum of the inguinal region which had a thickness of about 30µm, the diameter of hyphae was 3 - 4.5µm.

5:7 Conclusion:-

In the previous two chapters (3 and 4) arthrospore adherence to human corneocytes and germination in suspensions of human corneocytes with penetration of these cells by germ tubes was found to occur. In the present study the fungal growth has been followed using sheets of human stratum corneum. By not applying antiseptic measures dermatophyte-bacterial interaction was allowed to go on, thereby a picture near to the in vivo situation has been obtained. The data from this study and the previous two (Chapter 3 and 4) suggest that germination of arthrospores and penetration of stratum corneum by germ tubes are more important than adherence of arthrospores to corneocytes in establishing a lesion. In penetrating stratum corneum the fungus grows transversely and through the thickness of the stratum corneum and invades individual corneocytes. Therefore, fungal elongation can be regarded as a pathogenic factor in dermatophytosis. The intra and extracellular location of dermatophytes suggests that they are able to utilize components of both the intra and extracellular compartments.

CHAPTER 6

Dormancy and survival of arthrospores

6:1 Summary:-

Dormancy and survival of arthrospores were investigated. Under experimental conditions not suitable for germination arthrospores demonstrated an exogenous type of dormancy provided that these conditions were not lethal to the arthrospores themselves. Survival of arthrospores after 6 days incubation under conditions which resembled ordinary environmental ones was significantly more, P values were less than 0.05 and 0.01 in the presence of corneocytes than in their absence. The epidemiological significance of these findings is discussed.

6:2 Introduction

Dormancy and resistance are properties ascribed to spores in general. Sussman (1966) defined dormancy as any rest period or reversible interruption of the phenotypic development of an organism and distinguished two types of dormancy, constitutional and exogenous. In constitutional dormancy the development is delayed due to an innate property of the organism such as a barrier to the penetration of nutrients, a metabolic block or production of self inhibitors.

In exogenous dormancy the development is delayed because of unfavourable physical or chemical conditions of the environment. Constitutional dormancy does not allow development to proceed even when conditions are suitable for vegetative growth; some form of treatment (activation) with various physical or chemical factors is required. Included in these factors are temperature, moisture, light and various chemicals (Sussman, 1966). Alternatively, constitutionally dormant spores require a period of aging before they germinate (Deacon, 1984). Exogenously dormant spores germinate whenever environmental conditions become suitable to do so. Generally the sexual spores of fungi often show constitutional dormancy and the asexual spores commonly show exogenous dormancy (Deacon, 1984).

Resistance to adverse conditions is closely related to dormancy. In dermatophytes, arthrospores were reported to be resistant to chilling and freezing but susceptible to moderate heat (48 - 60°C) and desiccation (Hashimoto and Blumenthal, 1978). This susceptibility to desiccation was found to be improved when arthrospores were dried in the presence of an

exogenous protein (Hashimoto and Blumenthal, 1978). Therefore these authors suggested that cornified tissues offer a protective coating to arthrospores.

6:3 Aim of the study:-

The aim of this study was to categorize the type of dormancy arthrospores of dermatophytes express. Additionally the suggested (Chapter 1, Section 1:11) role of corneocytes in protecting arthrospores shed along with them from lesions was investigated. This study may help to understand the longevity thereby infectivity of arthrospores shed from lesions into the environment.

6:4 Materials and methods:-

6:4:1 Organisms and stock cultures:-

The three dermatophytes which have been used previously were employed in this study. Stock cultures have been described in Chapter 3, Section 3:4:1.

6:4:2 Production and preparation of arthrospores:-

Arthrospores were produced and prepared in PBS and distilled water to a final concentration of 1×10^7/ml as has been described in Chapter 3, Section 3:4:4.

6:4:3 Viability of arthrospores:-

The viability of arthrospores was checked by the method described in Chapter 3, Section 3:4:3.

6:4:4 Dormancy of arthrospores:-

Dormancy was studied using sheets of stratum corneum stripped from the leg of a volunteer and inoculated with 10ml of arthrospore suspension. The method of stripping of stratum corneum, inoculation with arthrospores and incubation in a moist chamber has been described in Chapter 5, Section 5:4:5.

Temperatures of 45°C and 4°C, moisture and nutrient type (stratum corneum) were tested for their effect on arthrospore germination.

6:4:4:1 Temperature:-

At each of the two temperatures (45°C and 4°C) 3 strips of arthrospore-inoculated stratum corneum were incubated for a period of 24 hr. At the end of this period arthrospore germination was determined using one strip. One of the remaining two strips was transferred to 37°C and incubated for a further 16 hr and the other was further incubated at the original temperature (45°C or 4°C) for a period of 16 hr. Arthrospore germination in these two strips was determined at the end of periods of further incubation.

6:4:4:2 Moisture:-

In the absence of moisture three strips of arthrospore inoculated stratum corneum were incubated at 37°C for 24 hr. One strip was evaluated for arthrospore germination at the end of that period. To the second 8 ml of sterile distilled water was run into the chamber and incubation was continued for a further 16 hr and the third was left dry for a further incubation of 16 hr.

6:4:4:3 Stratum corneum:-

Three pieces (4.5 x 1.5cm) of adhesive tape (Steri Drape) were spread with 10ml of arthrospore suspension and incubated in the presence of moisture at 37°C for a period of 24 hr. At the end of this time, one strip was evaluated for arthrospore germination, the second was applied to the skin repeatedly till it became non adhesive and reincubated at 37°C, in the presence of moisture, for a further period of 16 hr and the third was left without stratum corneum for a further incubation period of 16 hr at 37°C, in the presence of moisture.

6:4:4:4 Control conditions:-

Strips of stratum corneum inoculated with arthrospores of the various strains were prepared and incubated at 37°C in the presence of moisture for 16 hr. At the end of this period arthrospore germination was evaluated as described previously.

6:4:4:5 Determination of arthrospore germination:-

Tapes were heat fixed at 45°C for 10-15 minutes, stained with PAS, transversely (Appendix), mounted in Von Apathy medium (Difco) and covered with 50 x 22 mm cover glasses. Percentage of arthrospores which had germinated were determined microscopically by examining different fields. Each experiment was read in triplicate.

6:4:5 Survival of arthrospores:-

The survival of arthrospores after 6 days incubation at room temperature was studied both in the presence and absence of corneocytes. Environmental and desiccated condition were employed.

6:4:5:1 Preparation of corneocyte - arthrospore mixtures

Corneocytes were obtained from the sole of a volunteer by gently scraping the surface of the skin with a surgical blade. Scraped corneocytes were standardized by weight. 100ml of arthrospore suspension (1×10^7/) in distilled water was added to 0.1 gm of corneocytes and incubated under the conditions specified below. Control incubation of arthrospores was carried out without corneocytes under the same conditions.

6:4:5:2 Environmental incubation:-

Samples were left at room temperature for a period of 6 days. At the end of this period the number of arthrospores which might have germinated was determined by resuspending the samples in 1 ml sterile distilled water and preparing mounts on pieces of Steri Drape. Mounts were stained with PAS and examined microscopically. Other samples were used to determine the viability of arthrospores after the 6 days incubation. Each sample was resuspended in 1 ml Sabouraud's dextrose broth and incubated at 37°C on a rotary shaker (12 rpm) for 24 hr. Mounts were made on pieces of Steri Drape, processed for PAS staining and examined microscopically.

6:4:5:3 Desiccation:-

A second set of samples were placed in a desiccator containing a drying agent (self indicating silica gel) and incubated at room temperature for 6 days. The number of arthrospores which might have germinated during the

incubation period and the viability of arthrospores after incubation were determined by the methods which have been described in the previous section.

6:5 Results:-

6:5:1 Viability of arthrospores:-

Table 6:1 shows the viability of arthrospores. An arthrospore was considered to have germinated when a visible germ tube had developed.

6:5:2 Dormancy of arthrospores:-

Table 6:2 shows the results obtained under the various conditions.

6:5:2:1 Effect of temperature on arthrospore dormancy:-

At 45°C germination of arthrospore did not occur after 24 hr incubation. Similarly, no germination was observed in those samples which were incubated for 24 hr at 45°C then transfered to 37°C for a further incubation period of 16 hr and in those samples which were left at 45°C for a further 16 hr.
At 4°C arthrospore germination did not occur after 24 hr and after the further 16 hr incubation. In those samples which were incubated for 24 hr then transferred to 37°C for a further incubation of 16 hr germination of arthrospores occurred and was significantly more than the controls in all strains, P values were less than 0.02 and 0.01 for strain number 121 and 126 respectively and exceedingly less than 0.001 for number 4. Between the strains there were no significant differences.

6:5:2:2 Effect of moisture on arthrospore dormancy:-

Arthrospores did not germinate in the absence of moisture after 24 hr and a further 16 hr incubation at 37°C.
In those samples to which moisture was added then incubated for a further 16 hr at 37°C germination of arthrospores occurred (Table 6:2), but in strains 126 and 4 it was less than the controls and the differences were statisticallysignificant, P values were exceedingly less than 0.001. There were

Strain	Dormancy	Survival
121	96.77±1.31	96.22±1.22
126	96.66±1.56	96.77±1.31
4	96.33±1.33	97.11±0.99

Table 6:1: - Viability of arthrospores. Data are the mean ± SD of percentages of germinated arthrospores and were calculated from three assays; each was read in triplicate.

Factor	Experimental Factor	121	126	4
Temperature	45°C	0.00	0.00	0.00
	45°C → 37°C	0.00	0.00	0.00
	45°C → 45°C	0.00	0.00	0.00
	4°C	0.00	0.00	0.00
	4°C → 37°C	77.66±3.46	68.22±7.43	70.22±2.43
	4°C → 4°C	0.00	0.00	0.00
Moisture	$-H_2O$	0.00	0.00	0.00
	$-H_2O$ → $+H_2O$	70.44±5.98	44.66±3.94	30.66±4.00
	$-H_2O$ → $-H_2O$	0.00	0.00	0.00
Stratum corneum	-Corneocytes	1.00±0.47	1.00±0.47	0.55±0.49
	-Corneocytes → +Corneocytes	69.33±3.94	67.88±3.89	58.11±3.07
	-Corneocytes → -Corneocytes	3.22±1.93	1.88±1.09	0.77±0.62
Control	37°C	70.55±8.61	57.77±4.07	58.11±3.63

Table 6:2: - Dormancy of arthrospores under various environmental conditions. Data are the mean ± SD of percentages of germinated arthrospores and were calculated from three dormancy experiments; each experiment was read in triplicate.

also statistically significant differences between strains 121 and 126, 126 and 4 and 121 and 4, P exceedingly less than 0.001 for all.

6:5:2:3 Effect of corneocytes on arthrospore dormancy:-

At 37°C, in the presence of moisture very low levels of arthrospore germination occurred in the absence of stratum corneum after 24 hr and a further 16 hr incubation (Table 6:2). On those tapes which were used to strip stratum corneum after being inoculated with arthrospores and incubated at 37°C in the presence of moisture for 24 hr, germination ocurred after further incubation for 16 hr under the same conditions. The germination was comparable to that of the controls with the exception of that of strain 126 where it was significantly more, P less than 0.01. The differences between 121 and 4; and 126 and 4 were significant, P values were exceedingly less then 0.001 and less then 0.01 respectively.

6:5:3 Survival of arthrospores:-

Arthrospore germination was not observed in those samples which were examined immediately after incubation under the various conditions. This indicates that arthrospore germination had not occurred during the incubation periods.

6:5:3:1 Survival of arthrospores under environmental conditions in presence and absence of corneocytes:-

Under environmental conditions (room temperature without superimposed desiccation), the survival of arthrospores was more in the presence of corneocytes than in their absence (Table 6:3). The differences were statistically significant; P was less than 0.01 for 121 and 4 and less than 0.05 for 126. No significant differences were found between individual strains in the presence or absence of corneocytes.

6:5:3:2 Survival of desiccated arthrospores at room temperature in the presence and absence of corneocytes:-

Table 6:3 shows the survival of arthrospores desiccated at room temperature in the presence and absence of corneocytes. The survival of arthrospores was greater in the presence of corneocytes than in their absence. The difference reached a significant level in T. interdigitale strain 4, P was less

Strain	Environmental Conditions		Desiccated Conditions	
	Corneocytes	Dist H_2O	Corneocytes	Dist H_2O
121	66.33±2.35	23.33± 2.49	30.00±3.26	24.33±10.07
126	71.88±2.35	27.33±13.91	31.33±8.73	27.33± 1.24
4	70.00±1.63	28.00± 6.37	14.33±2.86	7.66± 1.24

Table 6:3: - Survival of arthrospores after 6 days incubation at room temperature under ordinary environmental and desiccated conditions in the presence and absence of corneocytes. Data are the mean ± SD of percentages of germinated arthrospores and were calculated from one experiment read in triplicate.

than 0.05. The differences between the strains both in the presence and absence of corneocytes were not significant statistically; with the exception of that between 126 and 4 in the absence of corneocytes, P value was less than 0.01.

6:6 Discussion:-

The present study investigated the dormancy of arthrospores of dermatophytes and their survival under desiccation and conditions comparable to those occurring naturally.

6:6:1 Dormancy of arthrospores:-

Freshly harvested arthrospores were found to germinate readily in Sabouraud's dextrose broth (Chapter 4). Hashimoto and Blumenthal (1977), in addition to a similar finding reported that, the poor germination rate of arthrospores in the presence of simple peptides or single amino acids was improved if the arthrospores were initially treated in distilled water at 25°C for 24 hr or at 45°C for 10 to 20 minutes. They referred to the freshly harvested arthrospores as dormant and the treated arthrospores as activated. Sussman (1966) defined activation as the application of environmental stimuli to induce germination. In the previous two studies (Chapter 4 and 5), freshly harvested arthrospores were found also to germinate readily in suspensions of corneocytes and sheets of stratum corneum without the need of any form of activation. As an alternative to activation in endogenously dormant spores a period of ageing is required before germination occurs (Deacon, 1984). The arthrospores which were used throughout this study were harvested from cultures incubated in 10% carbon dioxide in air for 10 days. Under similar cultural conditions, Bibel et al . (1977) reported that mycelia began to collapse and arthrospores to disarticulate after 7 days incubation. Thus the arthrospores which were used throughout this study probably had 2-3 days of further development, i.e. ageing. It was not certain whether this period was required by the arthrospores to age before they could germinate, but if so the arthrospores would be regarded as constitutionally dormant, however, even constitutionally dormant spores may have periods of exogenous dormancy imposed by enviroment factors (Sussman, 1966). In the present study it was found that arthrospores inoculated on sheets of stratum corneum and

incubated in the absence of moisture or at 4°C did not germinate but when samples were transferred to 37°C and moisture added, germination occurred. Additionally, when arthrospores were inoculated on tapes without strips of stratum corneum, germination did not occur, but when sheets of stratum corneum were held on the same tapes germination of arthrospores occurred after a further period of incubation. These findings indicate that failure of the arthrospores to germinate was due to an exogenously imposed dormancy because when conditions became suitable, germination occurred. Surviving a period of dormancy is a prerequisite for germination to occur when conditions change to suitable ones because when arthrospore inoculated sheets of stratum corneum were transferred to 37°C after being incubated at 45°C, germination has not occurred. Therefore, from an epidemiological point of view the findings of the present study suggest that for arthrospores shed from lesions to be able to cause new infections they should survive in the environment i.e. in a dormant state. Because fungistasis (soilstasis), antibiosis, competition and lysis are factors known to govern microbial survival in the environment (Griffin, 1972), this suggests that the dormant state of the arthrospores shed from lesions into the environment can be characterized as an exogenously imposed one.

6:6:2 Significance of arthrospore dormancy:-

Significant differences in the survival of arthrospores of the different strains under the various conditions of exogenous dormancy were found. The germination of arthrospores after a period of exogenous dormancy imposed by incubation at 4°C was significantly higher than controls; P values were less than 0.02, 0.01, respectively for strains 121, 126 and exceedingly less than 0.001 for 4. After a period of exogenous dormancy imposed by absence of moisture, with the exception of arthrospores of strain 121, the germination of strains 126 and 4 were significantly lower than the controls; P values were exceedingly less than 0.001 for both. The figures after a period of exogenous dormancy imposed by absence of nutrients (stratum corneum) were statistically not different from those of the control with the exception of strain 126 whose arthrospores had germinated to a level significantly more than the control; P was less than 0.01. These findings suggest that arthrospores after being dormant for sometime are likely to have either enhanced or decreased germination according to the condition which has caused the dormancy. This, apart from the failure of arthrospores to survive at 45°C is evident also from the increased germination after prior hydration (Chapter 4, Section 4:5:3; Chapter 5 Section 5:5:3). These findings may be of

epidemiological importance in the spread of dermatophytosis by indirect means. Arthrospores shed from lesions into the environment are likely to be subjected to different conditions imposing an exogenous type of dormancy. Therefore, depending on the nature of the environmental conditions, arthrospores are likely to have either an enhanced or decreased germination.

6:6:3 Protective role of corneocytes for arthrospores:-

The survival of both desiccated and undesiccated (i.e. incubated under environmental conditions) arthrospores of the three dermatophytes after 6 days of incubation at room temperature was greater in the presence of corneocytes than in their absence. In the undesiccated arthrospores the difference was statistically significant P values were less than 0.01, 0.05 and 0.01 for strains 121, 126 and 4 respectively, and in the desiccated arthrospores the difference was significant with strain 4; P less than 0.05. These findings indicate that the corneocytes have offered some form of protection to the arthrospores. The nature of the protective factor(s) has not been investigated. Hashimoto and Blumenthal (1978) reported that the survival of desiccated arthrospores was improved significantly when they were dried in the presence of an exogenous source of protein, i.e. powdered skimmed milk, albumin and gelatin. The stratum corneum on the whole is composed of protein, lipid and carbohydrate (Chapter 1, Section 1:4). It is therefore not certain whether the protein moeity acted singly as the protective factor or along with the other components. Additionally because lesions of dermatophytosis are scaly, corneocytes are shed in clumps, therefore they may contribute a mechanical coating for arthrospores situated between them. However, in the environment the integrity of this coat depends on the fate of the corneocytes. Blank (1952) reported that if pieces of cornified tissue were dried out they became very hard and brittle. Therefore it is likely that arthrospores dissociate from corneocytes exfoliated into a dry environment. In infected scale exfoliated into a wet environment such as floors of sport centres and communual bathing facilities, the corneocytes are likely to remain intact because it was reported that, when dry pieces of cornified tissue were allowed to absorb moisure they became soft and pliable (Blank, 1952). However in the environment, wet conditions are apt to support growth of microorganisms which may attack the exfoliated scale. Whatever the case might be, the ability of arthrospores to adhere to corneocytes (Chapter 3) seems to play a major role in their acquiring protective substance(s) and/or coating from the stratum corneum. The

mechanism by which substances in the stratum corneum protect the arthrospores is not known but exogenous proteins, powdered skimmed milk, albumin and gelatin have been suggested (Hashimoto and Blumenthal, 1978) to work by providing an additional wall layer thereby minimizing water loss from the arthrospores.

6:6:4 Significance of the protection of the arthrospores by corneocytes:-

This study has shown that in the presence of corneocytes the survival of arthrospores under environmental conditions is improved. Therefore it can be assumed that arthrospores in lesions may acquire some form of protection from the stratum corneum. Thus they are likely to remain viable in the environment for a length of time and spread the infection. Dermatophytes are known to survive for quite long periods of time, up to 5 years in hair and scales stored in the laboratories (Rosenthal and Vanbreuseghem, 1962; Dvorak et al ., 1968) and they have also been isolated from different places and personal items (Chapter 1, Section 1:7). However, because the exact morphological form, i.e. hyphae or arthrospores, has not been specified, longevity and survival in those reports cannot be attributed solely to arthrospores.

Under conditions of desiccation the survival of arthrospores in the presence of corneocytes was more than in their absence but the difference reached a significant level only in strain number 4; P less than 0.05. The findings indicate not only that corneocytes offered some form of protection to the arthrospores against desiccation but also the arthrospores were susceptible to desiccation. This susceptibility was evident from the statistically significant differences between the survival of arthrospores in the presence of corneocytes under undesiccated (enviromental) and desiccated conditions; P value were less than 0.01, 0.05 and 0.01 respectively for strains 121, 126 and 4. However it is not known whether the degree of desiccation which was employed in this study could be encountered naturally. The reason for the drop in viability may be the loss of water from arthrospores not only under the desiccated incubation but also under the environmental conditions because the viability of arthrospores which were incubated under environmental conditions in the absence of corneocytes, was not statistically different from that of the arthrospores which were incubated under desiccated conditions in the presence of corneocytes.

6:7 Conclusion:-

Arthrospores of dermatophytes express an exogenous type of dormancy under conditions unsuitable for germination, but not lethal to the arthrospores. It is not certain whether a period of ageing is required before they germinate, but if so they may also be classified as endogenously (constitutionally) dormant spores. However, it is known that even endogenously dormant spores express exogenous dormancy (Sussman, 1966).

The corneocytes improve the survival of arthrospores under environmental conditions. Therefore arthrospores shed from infected stratum corneum are likely to remain viable for some period of time in the environment. This corneocyte-mediated improvement of arthrospore survival substantiates their role in the indirect spread of infections.

CHAPTER 7

In vitro assessment of antifungal drugs using a corneocyte model.

7:1 Summary:-

The application of corneocytes as a model for the study of the effect of antifungal drugs on dermatophytes was investigated. Two phases of research were designed; the effect of antifungals on germination of arthrospores and the viability of arthrospores and germ tubes after exposure to the drugs. The results showed that corneocytes offered a simple, rapid, inexpensive and accurate model for the assessment of antifungal activity of drugs.

7:2 Introduction:-

The methods which are generally used to study the antimycotic effect of drugs in vitro are broth dilution and agar diffusion. Dermatophytes remain confined to the stratum corneum and reports on the use of stratum corneum (Knight, 1973, 1974) and excised human skin (Kligman et al., 1986) as models for the assessment of antifungal drugs against dermatophytes are few. It is known that dermatophytes are supplied with a battery of different enzymes which can act on various substrates including keratin (Chapter 1, Section 1:5) and antifungal drugs are thought to attack at distinct metabolic activities of dermatophytes. Griseofulvin, for example, acts on microtubule formation and clotrimazole on cell membrane synthesis. Therefore the use of the human target tissue of dermatophytes for in vitro testing of antifungal drugs against dermatophytes by simulating the parasitic growth of the fungus is thought to be more appropriate than laboratory media.

7:3 Aim of the study:-

The aim of the study was to investigate the possibility of the application of corneocytes as an in vitro model for the testing of antifungal drugs using the germination assay previously described. The effect of two antifungal drugs, clotrimazole and griseofulvin, on arthrospore germination and viability of germ tubes and arthrospores was studied.

7:4 Materials and methods:-

7:4:1 Organism and stock cultures:-

T. mentagrophytes strain 121 was used in this study. Stock cultures were described in Chapter 3, Section 3:4:1.

7:4:2 Production and preparation of arthrospores:-

Arthrospores were produced and prepared to a concentration of 1 x 10^7/ml in PBS as described in Chapter 3, Section 3:4:2 and 3:4:4.

7:4:3 Viability of arthrospores:-

The viability of arthrospores was checked by the method described in Chapter 3, Section 3:4:3.

7:4:4 Preparation of corneocytes:-

Corneocytes from the sole of a volunteer were obtained and prepared to a concentration of 1 x 10^6/ml in PBS as has been described in Chapter 3, Section 3:4:5.

7:4:5 Preparation of antifungal drugs :-

Clotrimazole and griseofulvin were employed. Solutions of the drugs were prepared by the method described by Warnock (1989). 64 mg of the drug was dissolved in 50 ml dimethyl sulphoxide (DMSO) and allowed to stand for 30 minutes to permit self-sterilization, then dispensed in 1ml quantities and stored at 4°C till used. To 1ml of the drug solution was added 9 ml of sterile distilled water to achieve a concentration of 0.128mg/ml which was used in the experiments.

7:4:6 Assessment of antifungal activity:-

Two parameters were used to assess the antifungal activity of the drugs: germination of arthrospores and viability of arthrospores and germ tubes. Two phases of investigation were designed: phase 1 consisted of determination of germination of arthrospores and viability of fungal

elements after incubation in a medium containing an antifungal drug, and phase 2 consisted of initially germinating arthrospores in a medium lacking antifungal activity, and then after adding an antifungal drug and continuing incubation for a further period, the germination of arthrospores and viability of fungal elements were determined.

7:4:6:1 Phase 1:-

The following mixtures were prepared:- (1) 0.3 ml of arthrospore suspension plus 0.3ml of the corneocyte suspension plus 0.6ml of an antifungal drug. (2) 0.3ml of the arthrospore suspension plus 0.3ml of Sabouraud's dextrose broth (SDB) (Appendix) plus 0.6ml of an antifungal drug. (3) For control purposes the antifungal drugs were substituted with DMSO and distilled water in mixtures similarily prepared as above. Mixtures were incubated at 37°C on a rocker (12rpm) for 16 hr. Arthrospore germination was determined microscopically by examining mounts made on pieces of an adhesive tape (Steri Drape) and stained with PAS (Section 4:4:5, Chapter 4). The viability of fungal elements was assessed by culturing on Sabouraud's dextrose agar (Appendix) and incubating at 28°C.

7:4:6:2 Phase 2 :-

Initially, arthrospores were germinated with corneocytes by adding 0.3ml of the arthrospore suspension to 0.3ml of the corneocyte suspension and incubating at 37°C on a rocker (12 rpm) for 16 hr. At the end of this period the percentage of arthrospore germination was determined from a control sample by the method described in Chapter 4, Section 4:4:5. To other samples, 0.6ml of an antifungal drug, substituted with distilled water and DMSO for the controls, was added. Incubation was carried out for a further period of 24 hr under the same conditions. Germination of arthrospores and viability of fungal elements at the end of 24 hr incubation were determined as has been described in phase 1.

7:4:7 Statistical analysis of results:-

The results were analysed by the Student's t-test.

7:5 Results:-

7:5:1 Viability of arthrospores:-

An arthrospore was considered to have germinated when a visible germ tube had developed. The viability of arthrospores was 96.66 ± 1.33, as calculated from three viability assays; each was read in triplicate.

7:5:2 Effect of antifungal drugs on arthrospore germination:-

In those samples which were incubated for 16 hr, i.e. phase 1, arthrospores had germinated under the control conditions (DMSO and distilled water) as well as in the presence of antifungal drugs (Table 7:1). However in the presence of an antifungal drug the germination of arthrospores in both corneocyte suspensions and Sabouraud's dextrose broth was significantly lower than in the control; P values were exceedingly less than 0.001 in all conditions. The difference between arthrospore germination in the presence of distilled water and DMSO in Sabouraud's dextrose broth was significant; P value was less than 0.01. In those samples which were incubated for a further period of 24 hr after the addition of antifungal drugs, i.e. phase 2, the germination of arthrospores (61.00 + 2.05 for clotrimazole and 60.88 + 1.72 for griseofulvin) (Table 7:2) was comparable to that after the initial incubation for 16 hr in the absence of antifungal drugs (62.22 + 1.81) (Table 7:2) and also to that in the corneocyte control samples of phase 1 (60.22 + 2.89 for DMSO and 62.44 + 2.45 for distilled water) (Table 7:1). In phase 2, the germination of arthrospores after the addition of distilled water was significantly more than that after the addition of antifungal drugs or DMSO and also more than that of the initial incubation for 16 hr; P values were exceedingly less than 0.001 in all conditions.

7:5:3 Effect of antifungal drugs on viability of fungal elements:-

All samples of both phases yielded positive results in culture.

7:6 Discussion:-

In suspensions of corneocytes, arthrospores were found to germinate (Chapter 4). In the present study the feasibility of corneocyte as an in vitro model for the testing of antifungal drugs was investigated. Two phases of study were designed, phase 1 and 2. In phase 1 the arthrospores in

Solution	Corneocytes	S D B
Clotrimazole	33.44± 7.84	44.22± 7.25
Griseofulvin	33.88± 8.53	41.88± 6.13
DMSO	60.22± 2.89	84.44± 2.45
Dist H20	62.44± 2.45	93.55± 2.91

Table 7:1: - Effect of antifungal drugs on germination of arthrospores. Phase I experiments: Antifungal drugs were added to the media from the start. Data are the mean ± SD of percentages of germinated arthrospores and were calculated from three assays; each assay was read in triplicate.

Solution	Germination
Clotrimazole	61.00± 2.05
Grisofulvin	60.88± 1.72
DMSO	60.55± 2.58
Dist H20	78.00± 2.94
PBS/16 hours	62.22± 1.81

Table 7:2: - Effect of antifungal drugs on germination of arthrospores. Phase II experiments: antifungal drugs were added after arthrospores germination has commenced. Data are the mean ± SD of percentages of germinated arthrospores and were calculated from three assays; each assay was read in triplicate.

PBS/16 hours = Incubation of arthrospores and corneocytes was done for 16 hours in PBS without antifungal drugs, DMSO or distilled water.

corneocyte suspensions or SDB were exposed from the start to antifungal drugs and in phase 2 arthrospores germinated in suspensions of corneocyte were exposed to antifungal drugs in the same suspensions. Therefore it is likely that phase 1 represented a prophylactic measure against an infection with a dermatophytosis and phase 2, treatment of an infection. The results of this study showed that the antifungal drugs, clotrimazole and griseofulvin, at the concentration in which they were used (0.064mg/ml) in phase 1 experiments reduced arthrospore germination up to significant level; P values were exceedingly less than 0.001. In phase 2 experiments germination was arrested at levels comparable to that of the incubation of arthrospore in corneocyte suspension for 16 hr in the absence of an antifungal drug.

In control samples of both phases arthrospore germination was more in those which contained distilled water than in those which contained DMSO and the difference reached significant levels; in phase 2, P was exceedingly less than 0.001. and in phase 1 with SDB, P was <0.01. Thus it seems that DMSO might have an inhibitory effect on arthrospore germination. Alternatively distilled water might either by diluting the arthrospore concentration enhance the germination and/or in the case of corneocyte suspensions increased the availability of water soluble substances to the arthrospores.

The positive cultures could not be attributed to a particular fungal form because at the end of each experiment in both phases germ tubes as well as arthrospores were present and it is not known whether those arthrospores which have not germinated were killed by the antifungal drugs or merely inhibited from germinating.

7:6:1 Corneocytes as an in vitro model to study antifungalactivity of drugs:-

Although arthrospores are the morphological forms of dermatophytes which are likely to play a major role in the spread of dermatophytosis, few studies (Hashimoto and Blumenthal, 1977, 1978; Scott et al., 1984) have investigated their sensitivity to antifungal drugs and these were conducted in laboratory media. In the present study the effect of two antifungal drugs, clotrimazole and griseofulvin, on arthrospores was investigated at two phases, firstly on the germination of arthrospores and secondly on viability of a combination of arthrospores and germ tubes. For phase 1 two media, SDB and a suspension of human corneocytes, were used and for phase 2 one medium, suspension of human corneocytes, was used. The germination of arthrospores in corneocyte suspensions was signficantly lower than in SDB

both in presence and absence of antifungal drugs; P values were less than 0.05 for the antifungal drugs and exceedingly less than 0.001 for the control. In a previous study (Chapter 4, Table 4:1) germination of arthrospores of three dermatophytes, two strains of T. mentagrophytes and one strain of T. interdigitale, in SDB was found to be significantly higher than in suspensions of corneocytes from palm and sole. Therefore it is clear that the nature of the media affects the germination of arthrospores. Because the target tissue of the dermatophytes is the stratum corneum (Chapter 1 Section 1:5) this model had a close resemblance to the in vivo situation of prevention and treatment of dermatophytosis.

7:7 Conclusion:-

Human corneocytes can be used as an in vitro model for the study of antifungal effects of drugs. A large scale study employing different individuals can be performed because the manner by which corneocytes are obtained is simple. For the basic and pharmacological development of antifungal drugs this model offers an inexpensive, rapid and accurate method and it may be used as an intermediary step between laboratory testing and clinical trials of drugs. Also it can be adopted to test for the incorporation of a systemically administered antifungal drug e.g. griseofulvin in the stratum corneum. This may be done by scraping the patients own corneocytes after a systemic treatment and using them in the germination assay.

CHAPTER 8

General discussion.

Arthrospore formation in vitro was studied under various cultural conditions: incubation temperature of 37°C, presence of antifungal drug in the media, elimination of glucose from the media and increased CO_2 tension in the incubation atmosphere. All of these conditions with the exception of the antifungal drug were found to be associated with arthrospore formation (Chapter 1). These conditions bear resemblance to factors present physiologically in the skin and/or become intensified pathologically e.g. by dermatophytosis. Since these cultural conditions were found to be, unlike the skin, not exclusive for the formation of arthrospores, it was suggested that, yet unidentified cutaneous factor(s) may be involved solely or in combination with them in arthrospore formation in vivo (Chapter 2, Section 2:6:5). Structurally, the stratum corneum is composed of multilayers of tightly packed corneocytes with a narrow well-filled intercellular space (Chapter 1, Section 1:4). Therefore it was suggested (Chapter 5, Section 5:6:3) that the arthrospores because they are produced by fragmentation of hyphae are the most suitable of the dermatophyte spores for the growth of dermatophytes in the stratum corneum, unlike micro and macroconidia which are formed laterally and apically on hyphae thereby requiring a space for their expansile type of development. Additionally, hyphae forming arthrospores on the stratum corneum appeared aseptate at some branches or terminal segments. This mode of growth helps the dermatophytes to escape the epidermal exfoliative capacity and at the sametime produce contagious propagules. Ultrastructurally the disarticulated arthrospores appeared mainly round, surrounded by a thick wall and were encircled by indentations. Adherence of an arthrospore to a corneocyte occured and depended on a close approximation between the walls of the two cells with the adherence site filled with floccular material. The nature and origin of the material, i.e. from the arthrospore wall, the stratum corneum or both, along with the mechanisms involved in the adherence require further investigation. Another point which needs studying is the influence of sebum on arthrospores adherence to corneocytes. In this study corneocytes from palm and sole were used in the adherence assays and both sites are devoid of sebaceous glands.

Germination of arthrospores, both adherent and non adherent, and the initial growth of germ tubes occurred extracellularly to the corneocytes indicating that both events were supported either by components of the extracellular space or components from the inside of corneocytes which had diffused extracellularly, or both. Alternatively, the arthrospores may contain endogenous food supply enough to support these two events before the full exploration of the cornified tissue by emerging germ tubes commences.

However, poor germination of arthrospores in phosphate buffered saline along with the development of short, thin and faintly stained germ tubes argues against the later alternative possibility being solely involved.

Over the emerging germ tube the arthrospore wall did not rupture but appeared thinned and there appeared to be influx of the arthrospore cytoplasm into the germ tube. With further growth germ tubes did not restrict themselves to the extracellular compartment but invaded the corneocytes. This mode of intra and extracellular location of germ tube may suggest the ability of dermatophytes to utilize components in both localities. For the utilization of intracellular components of corneocytes in particular, this was further suggested by the presence of clear, electron transluscent, zones around fungal elements located within the keratin of a scale from naturally occurring lesions (Chapter 5). In penetrating the stratum corneum, germ tubes grew transversely and through its thickness.

The improved survival of arthrospores incubated under environmental conditions in the presence of corneocytes (Chapter 6) shows that, the stratum corneum confers some form of protection to the arthrospores. This finding has an epidemiological significance by increasing the longevity and thereby the infectivity of arthrospores. The chemical nature of the protective agent and whether it is also conferred to hyphae needs to be established. The survival of arthrospores expressed itself as an exogenous type of dormancy provided the conditions which have imposed the dormancy were not lethal.

In conclusion the events involved in the colonization and invasion of the stratum corneum by dermatophyte fungi can be summarized as follows: adherence of arthrospores to corneocytes, germination of arthrospores, penetration of the stratum corneum by the germ tubes and arthrospore formation (Figure 8:1). In penetrating stratum corneum the germ tubes invade individual corneocytes, grow transversely and through the thickness of the stratum corneum and form branching hyphae. This type of growth lead to the development of fungal mass (mycelium) in the stratum corneum which diagramatically can be representd as shown in Figure 8:2. The epidermis is a proliferative tissue and the corneocytes are continuously exfoliated into the exterior. Thus, mechanically, dermatophyte growth is counteracted by the epidermis, the proliferation of which was in fact reported to be increased in dermatophytosis (Section 1:5, Chapter 1).

From the pathogenic point of view, adherence of arthrospores to corneocytes along with their germination and penetration of the stratum corneum can be regarded as mechanisms operative in the etablishment of dermatophytosis.

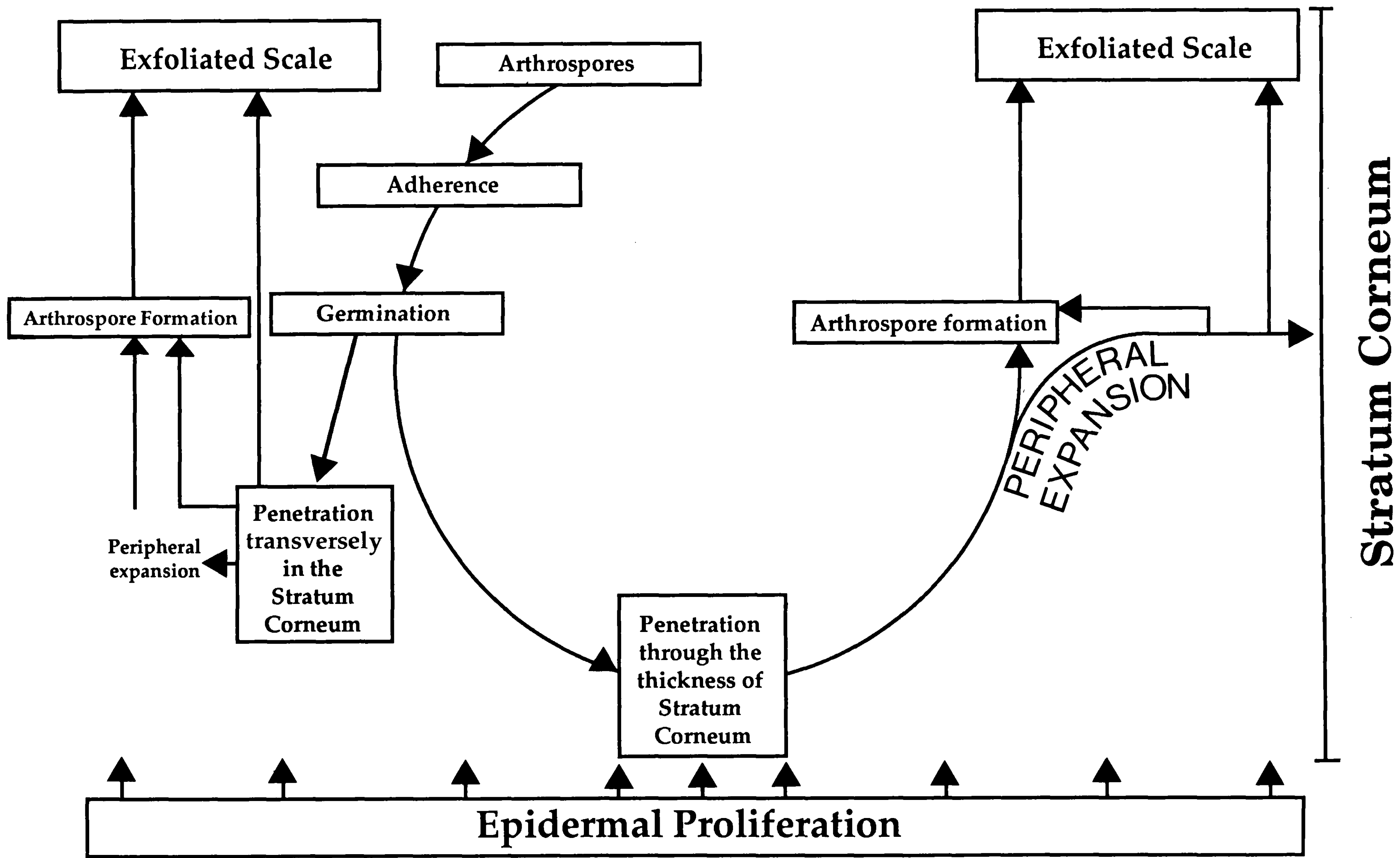

Figure 1:- Diagram of the events involved in colonization and penetration of stratum corneum by dermatophyte fungi

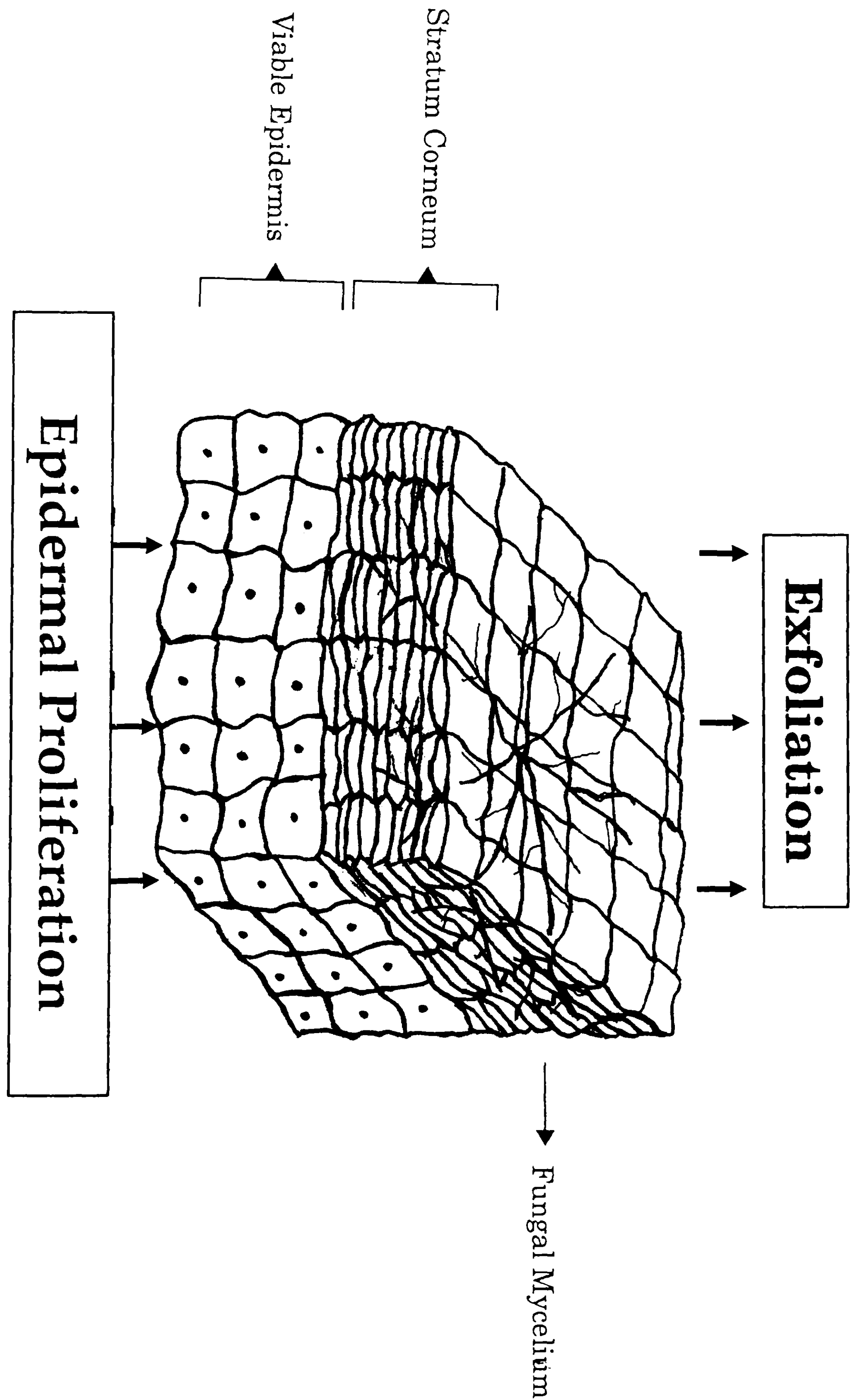

Figure 2 :-
Diagramatic representation of a dermatophyte branching mass (Mycelium) in Stratum Corneum. The epidermal proliferation and exfoliation procede in an opposite direction.

Appendix

I. Media

All media were sterilized	
at @ 15 lbs/in^2 for	20 min
Sabouraud's dextrose agar	
Composition/500 ml distilled water	
Glucose	20 gm
Peptone (Bacteriological, Difco)	5 gm
Agar (Bacteriological, Difco)	10 gm
Chloramphenicol stock solution	5 ml
Sabouraud's dextrose broth	
Composition/500 ml distilled water	
as above but without agar	
Peptone broth	
Composition/500 ml distilled water	
Peptone (Bacteriological, Difco)	20 gm
Chloramphenicol stock solution	5 ml

II. Chloramphenicol stock solution

Composition/100 ml 95% alcohol	
Chloromycetin	0.5 gm

III. Phosphate buffered saline (PBS)

Composition/litre distilled water	
Potasssium dihydrogen phosphate	0.34 gm
Di-Potassium hydrogen phosphate	1.21 gm
Sodium chloride	8 gm

IV. Periodic acid-Schiff's staining

The slides after being heat fixed at 45°C for 10-15 min were stained as follows	
Periodic acid	20 min
Distilled water	5 min
Schiff's reagent	20 min

The slides were mounted in Von Apathy medium (Difco) and covered with cover glasses (22 x 50mm).

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