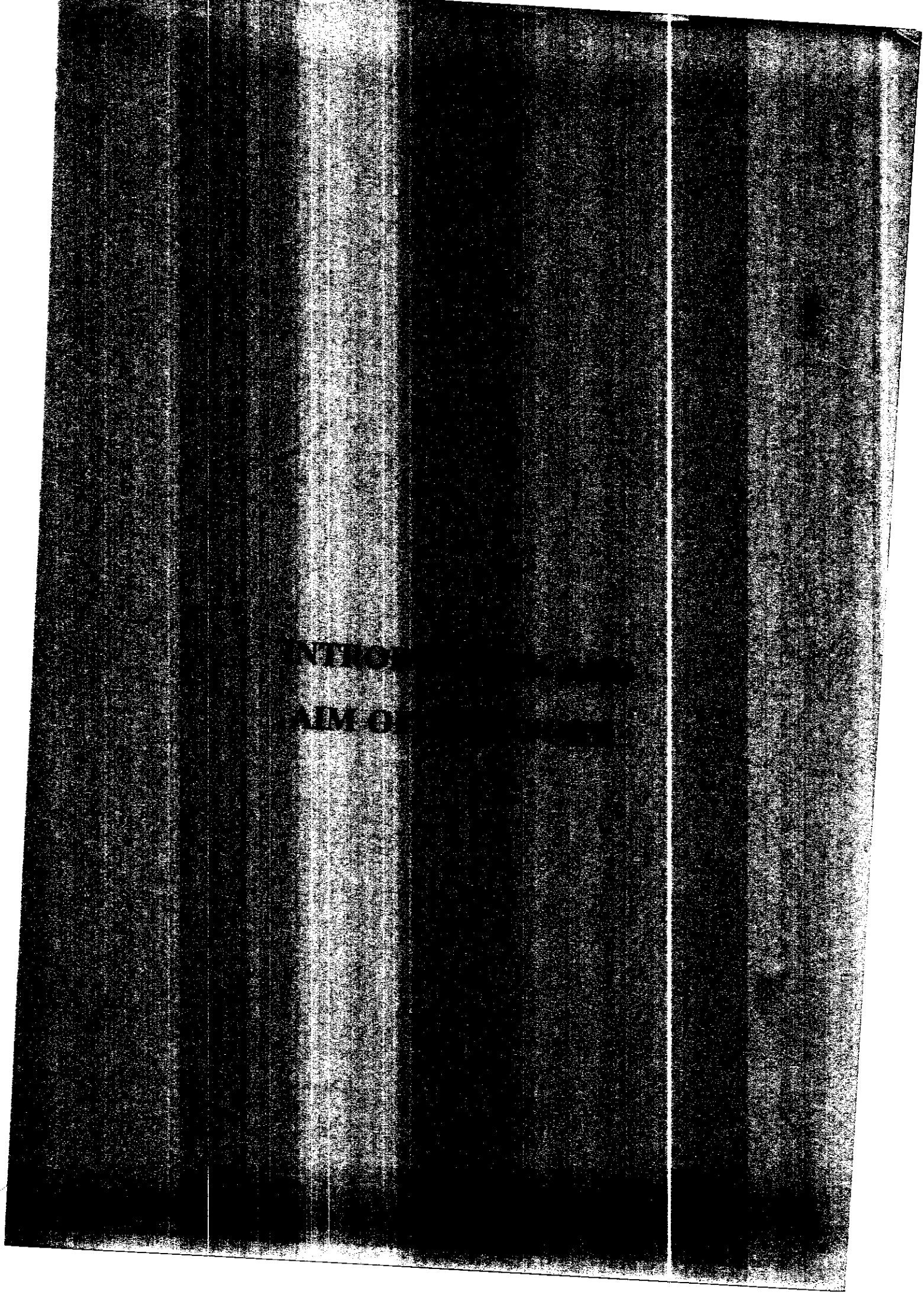


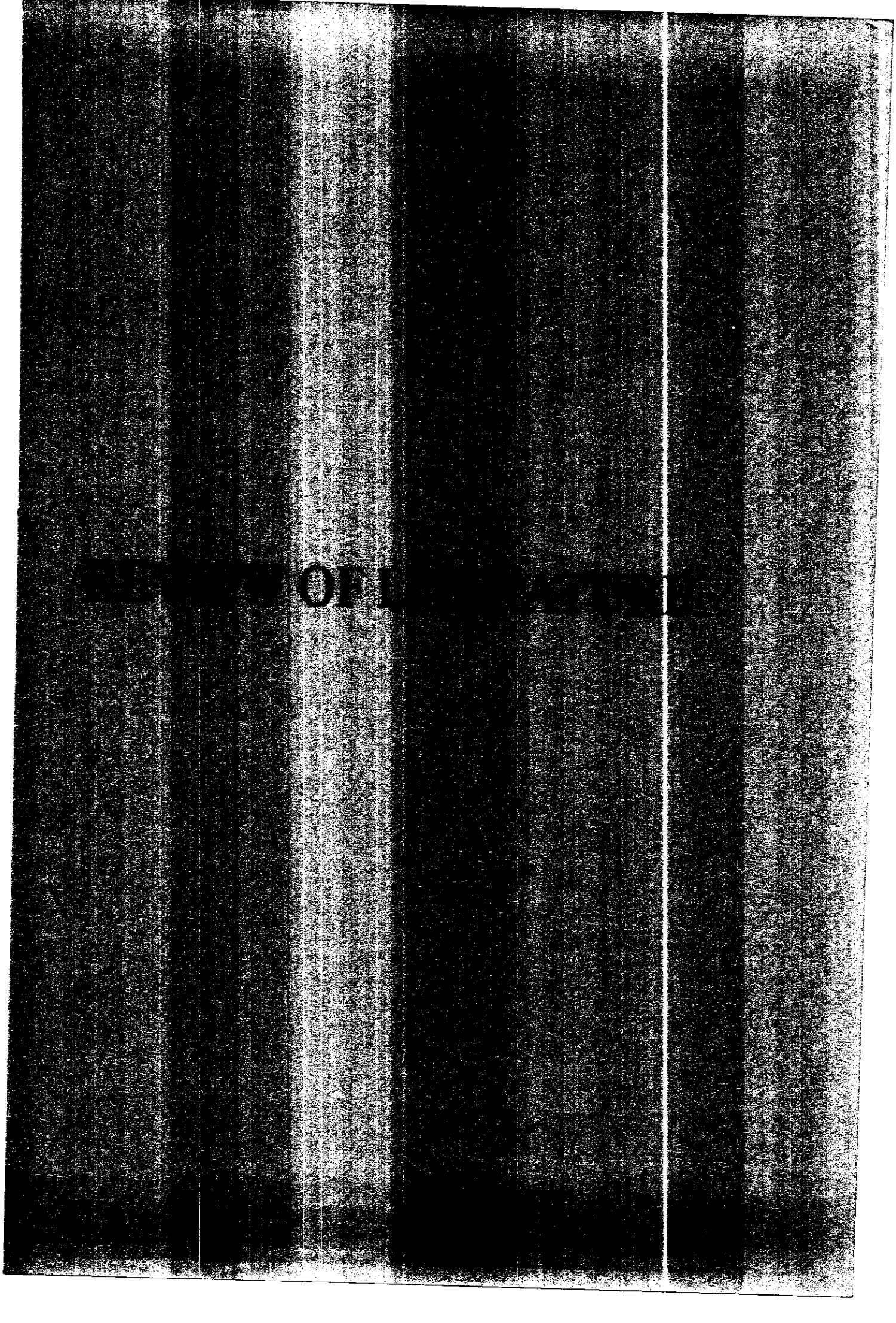
Dermatophytes are a group of filamentous fungi which are put under family Moniliaceae. They are closely related antigenically, physiologically and morphologically [Rebell and Taplin, 1970]. The term ringworm, tinea, dermatophytosis and dermatomycosis are sometimes used synonymously [Beare et al., 1972].

Dermatophytes may be pathogenic or saprophytic. Pathogenic members characteristically have the ability to invade the cutaneous tissue of animals and/or human beings but rarely penetrate to subcutaneous tissues or internal organs. These lesions are limited to hairs, nails and stratum corneum of skin and may involve small or large areas of the cutaneous tissue producing from mild cosmotic problems to debilitating and disfiguring diseases. The disease produced by these dermatophytes collectively known as the dermatophytosis. The saprophytic members of dermatophyte live in the soil and known as the soil keratinophils [Rippon, 1982].

Most resistance to dermatophyte infection depends on a number of factors ranging from proliferative response of epidermis to T-lymphocyte mediated immunity, non specific serum factors including transferrin may inhibit fungal growth in non-immune patient [King et al., 1975]. There is also evidence of an increased turnover of epidermal cells beneath an area of skin invaded by a dermatophyte which presumbly enhance shedding of organism [Berk, et al., 1976]. The role

played by a circulating antibody in defence against dermatophytosis is difficult to assess as they may be absent in a significant proportion of infected patients [Hay and Shennan, 1982]. Cell mediated immunity is generally considered as the principle immunological response to dermatophyte infection [Svejgaard, 1986]. The macrophages and T-lymphocytes involved in this cell-mediated reaction are located in the dermis and produce factor "lymphotoxin" that mediated inflammation and damage the epidermis overlying the infected area [Jones et al., 1974].

The aim of this work is to evaluate T.cell function in the pathogenessis of dermatophytes infection, by the E. Rosette method [Hudson& Hay, 1976], and lymphocyte blast transformation [Stites, 1987].



DERMATOPHYTES AND DERMATOPHYTOSIS

The dermatophytes are a group of toxonomically related fungi, capable of colonizing keratinized tissues such as the stratum corneum of the epidermis, nails, hair, horny tissues of various animals, and the feathers of birds. As a consequence of this predilection for keratin, these organisms are referred as the "Keratinophilic fungi" [Goslen and Kobayashi, 1987]. These fungi are among the commonest infectious agents of man, and no peoples or geographic areas are without "ringworm" [Rippon, 1982].

Dermatophytosis; superficial infections caused by dermatophytes, are characterised by many clinical manifestations e.g. The prominent feature of dermatophytic infections of skin, broken hairs, dystrophic nails and varying degree of inflammatory and eczematous reactions, this is due to the highly developed host parasite relationship [Bulmer and Fromtling, 1983].

HISTORICAL REVIEW OF DERMATOPHYTOSIS AND DERMATOPHYTES :

The term Porrigo was used for all diseases affecting the skin of the scalp. Tetters was the term for all other skin diseases affecting any part of the body. It was replaced later by the term Herps. Tinea was an old term used by Arab physicians, gradually replaced the term Porrigo (Rook, 1978).

The term tinea was also used by Romans, meaning any small insect larva. Also, the term referred to a group of keratinophilic insects e.g., the clothes moths (Ajello, 1974).

Ringworm was the popular term for any annular expanding lesion, it was used by many dermatologists (Rook, 1978) and it was a combination of Greek and Latin terms (Sabouraud, 1936).

Alphonse Devergie (1798-1879) accepted that ringworm is a contagious disease caused by production of mycelium and release of granules. Ernest Bazin (1807-1878) was one of the first dermatologists to accept the dermatophytes as pathogen; he published his monograph on ringworm reviving old term: tinea (Rook, 1978).

In 1834, Remak examined materials from favus and noted the presence of filaments resembling a mold. He failed to produce the disease on his arm by rubbing the organism on his skin. Schoenlein in 1839 described the filaments as being molds. David Gruby (1841) isolated the organism of favus on potato slices and succeeded in the production of disease on normal skin. Gruby (1843) named the fungus microsporum audouini to the fungus causing childhood ringworm refering the name to his professor Jean Victor Audouin (Rook, 1978).

The credit for discovering the fungus of favus was given to Johann Schoenlein by Robert Remak, 1845. He named it Achorion Schoenleinii while Lebert named it Oidium Schoenleinii in the same year. Also, in 1845 Malmsten erected the genus Trichophyton and described T. tonsuran. T. mentagrophytes was discovered by Charles Robin in 1847. Sabouraud in 1890 began his systematic and scientific studies on the dermatophytoses. He accumulated his work in the volume "Les Teignes" (1910). He adopted four generic names for dermatophytes. Microsporum, Trichophyton, Epidermophyton and Achorion. He published after Les Teignes, improving the methods of treatment and studying of fungi in the laboratory (Rook, 1978).

Emmons in 1934 redefined the dermatophytes according to botanic rules. He accepted the genus Achorion with genus Trichophyton as proposed by Langeron and Michevitch in 1930 (Emmons et al., 1970).

Conant (1934) proposed a botanic grouping based on colony characteristics and clinical diseases. Georg (1958) classified them according to the nutritional requirments. Ajello (1968) defined one Epidermophyton, 20 Trichophyton and 14 Mircrosporum species.

In 1959, Dawson and Gentles described the perfect stage of Trichophyton ajelloi. This lead to the rapid discovery of the ascomycetes form of many dermatophytes (Badillet, 1982).

DERMATOPHYTE CLASSIFICATION :

Dermatophyte classification using the features of spores (macroconidia) served eventually as a method of definition. Three genera are recognized according to the nature of macroconidia. Those of trichophyton are thin walled smooth and pencil shaped usually with four to six transverse septa. In microsporum the macroconidia are spindle shaped, thick walled and with five to twelve or more septa. The wall is roughened with pits or tubercle like prominences. In Epidermophyton the macroconidium is pear-shaped, broadened or rounded at its distal pole, thick walled and smooth with up to four septa (Findlay, 1979).

The dermatophytes represent 39 closely related species in these three imperfect genera : microsporum, trichophyton and epidermophyton. The perfect or sexual state has now been recognized for 21 of the dermatophytes. Cleistothecia, which are fruiting bodies or ascocarps, are formed through the conjugation of two compatible mating types and sexual spores or ascospores are formed within these structures. The two perfect (sexual phase) genera are Nannizia and Arthroderma in

the subdivision Ascomycetes. In general, Nannizia corresponds to the microsporum imperfect state and Arthroderma corresponds to trichophyton. No perfect state has yet been found for the genus epidermophyton. The existance of a perfect state for many of the dermatophytes has allowed a more defenite classification and identification of these closely related fungi [Goslen and Kobayashi, 1987].

Ajello et al., (1963) classified dermatophytes from the epidemiological point of view into 3 groups according to their natural host :

a) *Anthropophilic species* : are human pathogens and rarely affects animals.

b) *Zoophilic species* : normally animal pathogens but all of them are known to cause ringworm in man and infection is transmitted from animals.

c) *Geophilic species* : Normally soil inhabitant and may infect man.

According to Ajello (1978) the recognized dermatophytes are 39 in number, these include two species in the genus Epidermophyton, 16 in Microsporum and 21 in Trichophyton. (Moore and Jaciow, 1979) only 12 species of dermatophytes produce most of the dermatophytic infections in

the world. These are : Epidermophyton floccosum, Microsporum audouini, M. Canis, M. gypseum, Trichophyton concentricum, T. mentagrophytes variant granulare, T. mentagrophytes var. interdigitale, T. rubrum, T. schoenleinii, T. tonsurans, T. verrucosum and T. violaceum.

1) Morphology of Dermatophytes :

A. *Microscopic characteristics :*

According to Lewis et al., (1958) when specimens of a dermatophytes colony are examined, various vegetative and reproductive forms may be distinguished.

i. Vegetative Structures :

1) Mycelium, hypae and thalus.

2) Pectinate bodies.

3) Racquet mycelium.

4) Nodular organ.

5) Spirals.

6) Arthrospores.

7) Chlamydospores.

ii. Reproductive Structures :

Reproduction occur by sexual spores. These are classed as macroconidia and as microconidia according to size, shape and structure.

B. Pleomorphism :

Old culture of dermatophytes tend to become pleomorphic and most dermatophytes mycelia regularly undergo fluffy degenerative changes in laboratory culture, with loss of identifying aleuriospores and pigments [Emmons, 1934]. This change may occur rapidly as happens with culture of Epidermophyton floccosum but it may be delayed for weeks or monthes in some species.

Some dermatophytes tend to become pleomorphic from primary culture. These changes have been shown to be due to mutation and chromosomal abberation [Weitzman, 1964].

2) Cultural Characters of Dermatophytes :

1. Temperature, O_2 requirment, PH :

Dermatophytes grow poorly at 37°C. The optimum range of temperature from 26-28°C. This factor alone may be responsible for lack of deep invasion by this group of organsims [Robert and Mackenzie, 1979].

Dermatophytes grow well in aerobic atmosphere, and low oxgen tension reduce their growth. As regard pH. they tolerate a wide range of pH from 4-10 [Lewis et al., 1958].

2. *Moisture :*

As regard moisture, the water requirment of fungi vary considerably according to the species. Most pathogenic species of fungi grow best on semi-solid media. Fungi may remain dormant for years under artificial or natural conditions of drying, being capable of revival with the addition of moisture. It is well known that growth on low humidity is slow [Lewi et al., 1958].

3. *Light :*

As regard light, the growth of dermatophytes has been almost equal in absolute darkness, subdue light and bright light. Certain ultraviolet rays have an inhibitory but not lethal effect. Roentgen rays and radium have had little action as lethal agent although inhibitory action on certain fungi have been reproted [Lewis et al., 1958].

4. *Nutritional requirements :*

Dermatophytes do not usually have very complex nutritional requirement but will not grow in absence of organic nitrogen source, so, the mixture of amino acid provided by the keratin of the skin is very suitable as a nitrogen supplement of dermatophytes. In culture media the usual source of nitrogen is peptone which

contains inorganic compounds, variable amount of amino acid. Certain species were found to syntheteize pigment in the presence of certain monosacharides of closely related structural formula such as dextrose, levulose and mannose. Pigment was not produced when the only sugar in culture medium was galactose (a monosaccharide) and other disacharides, trisacharides and polysaccharides [Lewis et al., 1958].

Vitamin requirement are variable, trichophyton verrucosum will not grow in the absence of thiamine. While the species which do not have absolute requirement for this vitamin may be stimulated by its presence [George and Comp, 1957]. Trichophyton rubrum and trichophyton mentagrophytes are autotrophic. Biotin occasionally stimulate the growth of microsporum. Trace of minerals such as ammonium, calcium, zinc, iron, copper, manganese, phosphorus and sulpher are needed for growth and reproduction [Lewis et al., 1958].

Media used for cultivation of dermatophytes :

The following media are used for cultivation of dermatophytes :

a) Dextrose agar [Lewis et al., 1958].

b) Modified dextrose agar [Lewis et al., 1958] :

If the specimen to be cultured is removed from a grossly contaminated site, or if there is difficultly in obtaining a pure fungus colony without coincident bacterial or mold growth, the addition of a selectively antibiotic is advisable as penicillin (20 unit per cubic centimeter (PCC), streptomycin (40 unit PCC) and chlormycetin (0.125 mg PCC) are effective against species of bacteria. Cyclohexamide (actidione) is added to suppres the growth of airborn mold..

c) Conservative agar : [Lewis et al., 1958] :

It was advocated by Sabouraud to prevent loss of character of the colony through excessive vegetative growth and minimise the chance of pleomorphism. The medium is similar to that of ordinary dextrose agar but without dextrose. The colony is neither too luxuriant nor too scanty and characteristics of the culture remain constant.

d) Potato dextrose agar [Al-Doory, 1980] :

It is used to stimulate sporulation in molds and enhance pigment production by dermatophytes.

e) Corn meal agar [Al-Doory, 1980] :

It is useful in studying the spore form of dermatophytes.

f) Rice grain agar [Al-Doory, 1980] :

It is used to assist in identification of microsporum species when microscopy is inconclusive.

Two new primary media have been developed which are very helpful in primary isolation of dermatophytes. They are both used on pH change caused by the proteolytic activity of dermatophytes which is lacking in most saprophytic fungi [Rippon, 1982]. The first is the ink blue agar of Baxter [Baxter, 1965]. The agar is blue but a colorless area appear around the growing dermatophytes colony. The second is dermatophytes test media [Taplin et al., 1969], the value of that media lies in its ability to inhibit bacteria and saprophytic fungi which allows dermatophytes to produce alkaline metabolities that changes the phenol red indicator from yellow to red.

3) **Antibiotic Production by Dermatophytes :**

Uri et al. (1957) recorded the production of antibiotic (penicillin) by dermatophytes in vivo, while Smith and Marples (1965) demonstrated its production in vitro and they reported that all producers were either trichophyton mentagrophytes or Epidermophyton floccosum, and that non producer were all trichophyton violaceum. Uri et al. (1957) demonstrated penicillin production in fragments of skin or hair from 21 of 25 patients infected with

dermatophytes. Youssef et al. (1978) described the production of commonly used antibiotic by dermatophytes fungi, these included, penicillin, 6-aminopenicillanic acid, and streptomycin-like antibiotic as well as a number of unclassified substances, produced by representatives of the species T. mentagrophytes, T. rubrum and E. floccosum.

Youssef et al. (1979) demonstrated the production of antibiotic (pencillin) in vivo and described the natural effect of this on the skin flora.

4. Pathogenesis of Dermatophytosis :

There are several stages through which the infection progresses. After inoculation of host skin with fungal organisms under suitable conditions as increased hydration of the skin with maceration (Hernandez, 1980). The stages include a period of incubation and then enlargement followed by a refractory period and a stage of involution (Rippon, 1982). During the incubation period the dermatophyte grows in stratum corneum, sometimes with minimal clinical signs of infection. A carrier state has been postulated when the presence of a dermatophyte is detected on normal skin by KoH examination or culture (Knudsen, 1975). Once infection is established in the stratum corneum, two factors are important in determining the size and duration of the lesion. These are rate of growth of

the organism and the epidermal turn over rate. The fungal growth rate must equal or exceed the epidermal turn over rate or the organism will be shed quickly [Hernandez, 1980].

Dermatophytosis are worldwide in distribution and differ from place to place, their prevalence is governed by the enviromental conditions, personal hygiene and individual susceptibility (Rippon, 1982).

As regards the role played by mechanical trauma, it was suggested that trauma is more effective in facilitating the minor continous friction, (Abdalla, 1971).

All dermatophytes are keratinophilic, but different genera vary according to the kind of keratin they prefer. Members of the genus Microsporum seem to prefer only the keratin of skin and hair and consequently they rarely invade nails. Epidermophyton species invade skin and nails, but do not attack hair. Trichophyton species are not fastidious, and will live on keratin of hair, skin or nails [Myrvik and Weiser, 1988].

Keratinases and other proteotytic enzymes are produced by dermatophytes (Meevootisom ans Niederpruem, 1979). The role of these enzymes in the pathogenesis of clinical

infection is not totally understood. There is an evidence that actual enzymatic digestion of keratin may be occuring [Verma, 1966]. It was found that zoophilic species are producing more proteolytic enzymes than anthropophilic one i.e. T. mentagrophytes and T. violaceum have a higher proteolytic activity than T. rubrum and Epidermophyton floccosum, it may be related to the more inflammatory lesion produced by them (Minocha et al., 1972).

The patterns of growth of the dermatophytosis in clinical picture (e.g.), in the skin, the infection spreads centrifugally showing the classic "ringworm" pattern. The host reaction may be limited to just scaly patches, toxic eczema form eruption or even inflammatory reaction may occur (Beare, 1972). Annular growth are produced displacing hyperkeratotic papular or vesicular borders with central clearing. It may be due to epidermal turnover and may result in limiting dermatophyte infection through the process of shedding; the central area would tend to revert to normal (healed) leaving a clinically cleared zone compared with the still active rim (Berk et al., 1976).

Infection of the nail occurs mostly at hyponychium resulting in hyperkeratosis, raising nail plate and loosening its structural integrity leading to soft crumbly

nail with hyperkeratotic nail bed. Infection may also occur in surface of the nail plate (white superficial onychomycosis) or within the nail plate itself giving rise to complete nail dystrophy (Hernandez, 1980).

In ectothrix tinea capitis; infection is confined to growing cycle (anagen) rather than the resting cycle (telogen) or (club hairs) so infection can persist as long as hair is actively growing. The infection begins in the stratum corneum spreading along the scalp. The hyphae produced, surround the follicular orifice, descend the hair's surface, becoming arthrospores and penetrate the hair cuticle at about mid portion of the follicle, then penetrate into hair cortex, branch and descend to the border of the keratogenesis zone forming "Adamson's fringe". The hyphae segment into arthrospores which are carried out upward on the surface of the hair forming ectothrix spores giving rise to gray, lusterless appearance of hair (Beare, 1972).

In endothrix infection of tinea capitis the mycelium does not break out on the surface of the hair but it remains inside and fragments into chains of larger arthrospores, filling the entire hair shaft replacing the structural stability of the hair, but in the lower portion of the follicle the hair are supported by the internal

root sheath so they are broken just below the surface resulting in black-dot tinea capitis (Beare, 1972).

The host's role in dermatophytes infections is undoubtedly additive to the above points and should be discussed Separately.

5. Immunity to Human Dermatophytes Infections :

Human infection is the result of a complex interplay of factors pertaining to the invading organism, the host, and the environment. This is best shown in human dermatophyte infections. Acute infections are usually short-lived and an easy to treat. The patient has good cell-mediated immunity, short term antidermatophyte antibodies, and delayed hypersensitivity. In chronic infections, the infection is long term and resistant to therapy. Patients have poor cel-mediated immunity and immediate hypersensitivity to fungal antigens [Ahmad, 1982].

FACTORS AFFECTING HOST RESISTANCE TO DERMATOPHYTOSIS :

A number of factors have been shown to affect the course of dermatophyte infections or inhibit growth of the fungus. In some instances resistance does not depend on prior sensitizations. These resistance factors in the nonimmune individual include serum, epidermal proliferation, and phagocytosis which known as non specific factors.

However, the development of a heightened response to a second infection indicates that sensitization may also lead to resistance. Both B & T lymphocyte-mediated responses have been implicated in immunity to dematophytosis, (Stone, 1985).

NON SPECIFIC FACTORS AFFECTING FUNGAL INFECTION :

1. Epidermal barrier.

2. Serum :

Serum contains a heat-labile dializable factor [Roth, 1959] that is inhibitory to the growth of organisms on skin [Blank, 1959]. The nature of this substance(s) is unknown, but it is present in nonimmune serum. One such factor is believed to be transferrin [Hay, 1983] unsaturated transferrin is inhibitory to growth of dermatophytes in vitro. This effect can be reversed by the administration of iron, and the mechanism may be a competitive one involving iron essential for hyphal growth. Transferrin is present in low concentrations in sweat as well as in serum and can be elaborated by lymphocytes [King, 1975].

3. Fatty acids in sebum :

The alteration in the fatty acid composition of sebum at puberty may explain the spontanious cure of tinea capitis at this time [Beare et al., 1972]. The effect of fatty acids on fungi is complex, for instance, medium

chain fatty acids stimulate the growth of pitrysporum species. However, the shorter chain compounds are inhibitory to the growth of dermatophytes in vitro, and it has been suggested that these compounds have also an inhibitory mechanism in vivo [Abraham, 1975].

4. Epidermal proliferation :

The epidermis is continuously desquamating, any mycotic infection that is confined to the stratum corneum must continuously invade deeper into it in order to avoid the process of desquamation, any factor that induces increased epidermal turnover would favor the host [Berk et al., 1976]. The mechanism that increase epidermal turnover is unclear. Likely, antigens diffusing down from the stratum corneum into the dermis and the subsequent immunologic response induce inflammation to play a definitive role. Thus, in the absence of cell-mediated immunity, infection can persist. When cell-mediated immunity established itself and intense inflammation occurs, then the dermatophyte is shed [Sohnle and Kirkpatrick, 1978].

5. Complement :

Complement can be activated by IgG or IgM after antibody interacts with specific antigen and can be activated by an alternative pathway by interaction of the cell wall constituents with complement components that

stabilize C_3 convertase. Activation of complement perhaps damage cell walls directly or induce inflamatory cells to injure the invading fungus [Swan et al., 1983]. The role of complement in host defense against dermatophytic fungal infection is uncertain complement may play no role when the infection by dermatophytic fungi is confined to the stratum corneum. If the fungus invades into the epidermis complement has access to the dermatophyte, and complement activation can occur. This activation and the resulting influx of polymorphonuclear leukocytes may stop fungal invasion [Dahl and Carpenter, 1986].

SPECIFIC IMMUNE RESPONSE TO FUNGAL INFECTION :

I. Humoral immunity :

Antibody is formed during the course of dermatophyte infection in animal and human [Hay, 1985]. In human infections the prevalence of antibody may be low. Although they can be demonstrated in the majority of patients with favus [Grappel et al., 1972] or tinea imbricata [Hay et al., 1983], both of which are chronic infections. The detection of antibodies varies with the methods used, and some normal, non infected individuals may also have antibodies to the organism [Hay, 1985]. In human infections, all classes of antibodies appear to be raised [Kaaman, 1981]. The detection of antibody in serum may be affected by two factors. First, it has been shown that

some antibodies to dermatophyte fungi have an affinity for epithelial tissue [Hopfer et al., 1975]. Second, some dermatophyte antigens may share common features with human antigens. For instance, T. mentagrophytes contains a glycoprotein that cross-reacts with human isoantigen A [Young and Roth, 1979]. It is possible that this allows the organism to evade detection because it may be identified as "host" [Hay, 1985].

Specific IgG antibodies towards dermatophytes are found in 90% of patients with inflammatory follicular dermatophytosis, in 62% of patients with tinea pedis and in 25% of patients with chronic T.rubrum infection. The results of follow-up investigations indicate that the antibody formation is transient in patients with acute lesions and persistant in those with chronic lesions. Specific IgE antibodies towards T.rubrum are present in patients with chronic T.rubrum infections [Svejgaard, 1986].

It has been shown that patients with dermatophytes infection caused by Trichophyton, Microsporum and Epidermophyton produce IgM antibodies that bind to intracellular substances. Antibody is seen in acute and chronic infection, is present in low titer and is removed from

serum samples by absorption with fungal myeclium (Ahmed, 1982). Enzyme-linked immunosorbent assay (ELISA) was used to determine the occurance of circulating antibodies to the enzyme keratinolytic proteinase (KPase) which is an important factor in the pathogenesis of dermatophytosis [Lee et al., 1988]. [Seeliger, 1962]) pointed out that serological techniques have not been of value for diagnostic purposes due to the cross-reactivity of some dermatophyte antigens with those of other microorganism. however, as purified antigens which are more specific for the dermatophyte group become available, rapid, sensitive serological tests may prove to be useful.

II. Cell-mediated immunity :

Resolution of fungal infection is usually associated with development of delayed hypersensitivity to the infecting organism [Jones et al., 1974]. The cell mediated immunity against trichophyton can be evaluated by the trichophytin intradermal skin test, in which a minute amount of purified trichophyton antigen (trichophytin) is injected intradermally and the injection site observed after 30 minutes and again after 48 hours. If delayed hypersensitivity to this antigen exists, the skin test site becomes inflammed and indurated within 48 hours. If delayed hypersensitivity is absent, no inflammation or induration is seen [Dick, 1979].

Delayed hypersensitivity to trichophyton antigen often develops after infection with dermatophytes. This has been studied by infecting volunteers. Different people react differently, but the usual response documents development of a cell mediated immune response [Hanifin et al., 1974]. If a person who has not previously had trichophyton infection is experimentally infected with trichophyton mentagrophytes, the infected site appears slightly inflammed with scales but is relatively asymptomatic. At this stage the 48 hours trichophytin skin-test reaction is negative. Between one to five weeks after infection the infected sites suddenly become intensely inflammed and pruritic. Trichophytin skin-testing at this time is positive. Delayed hypersensitivity has developed to trichophyton antigens. Following development of delayed hypersensitivity, lesions stop enlarging, cultures often fail to isolate fungus, and spontaneous resolution of infection usually occurs [Jones et al., 1974].

If a second infection is produced on a previously infected person who had a positive trichophytin skin test, the site of infection become intensely inflammed within two days. The infection does not spread or enlarge, [Kerbs et al., 1977].

The mechanism of cell mediated immunity to fungal infection. Antigens diffuse down from the stratum corneum and provoke primary sensitization, followed by clonal proliferation of lymphocytes capable of reacting against trichophyton antigen. These lymphocytes interact with antigen from the skin surface and elaborate inflammatory mediators. It is unclear just how inflammatory mediators are able to stop dermatophytic infection. Since the lymphocyte does not have access to the stratum corneum where the fungus is growing, a direct cytotoxic effect by lymphocytes on fungi is unlikely. Soluble mediators may be involved. The effect may be non-specific. Inflammation could increase the speed of epidermal turnover so that the infecting organisms are shed in the exfoliated scales; alternatively, specific cytotoxic factors elaborated by lymphocytes might be involved, or the oedema produced by the inflammatory response may allow a serum factor to exert an antimicrobiol effect [Jones et al., 1974].

HYPERSENSITIVETY TO DERMATOPHYTOSIS (TRICHOPHYTIN REACTION):

The "trichophytin" reaction is the term used for cutaneous hypersensitivity to dermatophyte antigens injected intradermaly in humans or experimental animals. Both immediate and delayed-type reactions occur. But the latter is most often associated with the resistance to reinfection. Immediate hypersensitivity appears in chronic infections, especially those due to T.rubrum. The cutaneous reactivity to "Trichophytin" becomes apparent 7 to 10 days after infection. After healing, the reaction remained positive for up to 3 years. The "Trichophytin" reaction is characterized by a lentil shaped, large, papule with an erythematous halo at 24 hours after which it decreases [Grappel et al., 1974].

There is a temporal correlation of lymphocyte blastogenic responses to trichophytin, positive skin test responses and intensification of erythema of lesions during dematophyte infections. Immediate type reactions to "trichophytin" have been found to be exceptionally prevalent in patients infected with T.rubrum. However, this immediate type urticarial reaction occured both in patients infected by other dermatophytes and in non infected individuals [Grappel et al., 1974]. A series of patients with urticaria, angioedema, asthma and rhinits who demonstrated immediate positive skin tests to intradermal trichophyton extract was identified. Most skin test positive individuals have IgE mediated hypersensitivity to trichophytin [Platts-mills et al., 1987].

The commercially available "trichophytin" preparations are mixture of crude extracts from dermatophytes and vary in their composition. Purified antigens are more sensitive than commercial materials. There is no firm relationship between infecting species and the species from which the antigen extracted. Furthermore, the proportion of dermatophytosis patients exhibiting delayed cutaneous reactivity to trichophytin was low, reaffirming the diagnostic limitations of this procedure [Hanifin et al., 1974].

TESTS FOR T-cell FUNCTION :

Several tests that are of value in assessing T-cell function available for clinical use. These include :

1. *Lateral roentgenograms of the chest :*

   To demonstrate the thymic shadow. Retrosternal radiolucency suggest thymic deficiency [Hong, 1979].

2. *Peripheral lymphocyte count :*

   Normally, more than 1500 lymphocytes/mm^3 are found in the peripheral blood. Marked drop in this count may suggest immune derangement [Hong, 1979].

3. *Skin tests :*

   a) Delayed hypersensitivity skin test to common antigens [Palmer & Read, 1974].

Several antigens are known to induce a delayed hypersensitivity skin reaction when injected intradermaly. These in common use are purified protein derevative (PPD), candida albicans and mumps. Failure to react to these antigens suggests, but not concludes, impairment of cell-mediated immunity.

b) Skin reaction to phyto haemagglutinin (PHA) :

PHA is a lectin derived from the kidney bean (Phaseolus rulgasis). It is predominantly stimulates T. cells. Normal subject respond, without perior sensitization to an intradermal injection of PHA with erytherma and induration at 24 hours. It has been used as a simple test for cell-mediated immunity in children [Douglas et al., 1978].

c) Contact Sensitivity :

Certain chemical e.g. : Dinitrochlorobenzene (DNCB), when applied to the skin result in T. cell sensitization. After a period of 2-10 days, challenge dose elicits a skin reaction of the delayed type. Failure of sensitization indicates a deficiency in cell mediated immunity [cataloma et al., 1972].

4. *T. lymphocytes count :*

This can be performed by one of the following technique :

a. E.Rosette-Forming cells (E.R.F.C.) : (Jontal et al., 1972) :

T-cells have got natural receptor (CD_2 molecule) for some animals red cells such as sheep red blood cells (SRBCs). Incubation of lymphocytes with these cells in a refrigerator (4°C) for at least 18 hours lead to the formation of rosette, so that the resulting count indicates T-cell number.

Modification of the E.Rosette test called the active E.Rosette. This modification consists of mixing SRBC with lymphocytes only for a brief period during centrifugation and measures "active E.rosettes". The latter are formed by subpopulations of T cells which have "high affinity" receptors for SRBC and which may be altered in number or in their capcity to rosette with SRBC in disease states.

Thus, both the determination of total E.rosettes (long term incubation 4°C) and active E.rosettes (brief incubation) may be clinically significant (Henry, 1984).

b) Immunofluorescent technique : [Stites, 1987] :

Anti human-thymocytes antisera prepared in rabbit is incubated with lymphocytes. This is followed by the addition of fluorescent compound resulting in a sensitive tracer with unaltered immunologic reactivity.

Immunofluorescent is essentially a histochemical or cytochemical technique for detection and localization of antigens.

5. **Blast transformation** [Stites, 1987] :

The stimulation of T.lymphocytes by a non specific mitogen like phyto haemagglutinin (PHA) results in a sequence of events called lymphocyte transformation with the production of blast like cells which synthesize DNA de novo. The extent of transformation can be quantified morphologically on the basis of change of typical small lymphocytes to larger lymphoblasts in which the nuclei become enlarged and euchromatic and develop one or more nucleoli. Also, their is increase in cytoplasmic basophilia and vacuolization.

Following antigenic stimulation small lymphocytes differentiate into larger blast cells that subsequently give rise to T.lymphocytes specific cytotoxic factor for CMI cells that regulate immune response. The degree of stimulation is measured either by the percentage of blast-like cells or by quantitation of incorporated labelled thymidine added to the culture.

6. **Tests for lymphokine production :**

Stimulated T.lymphocytes liberate a number of lymphokines. Their release into cellular medium indicates

a normal T.cell function. They can be identified by their effects.

The migration-inhibition tests is the most popular of these tests. Normally, leucocytes migrate out from cappillary tube in the form of "Fan Fashion". In peripheral blood Leucocytes are widely used cells as they provide both the responder cell (T.lymphocytes) as well as the indicator cells (polymorphs and macrophages) [Thompson, 1977]. Migration inhibition factor is the first lymphokine described. It is a soluble factor, the antigen induced production of migration-inhibition factor require a state of invivo hypersensitivity of the host to that antigen. However, mitogens such as PHA can induce its release in culture without prior exposure [Rocklin, 1978].

7. Tests for cytotoxicity :

It has been demenstrated that an allogenic grafts lead to the generation of population of cytotoxic or killer T.cells (K) in the recipient. The killer cells are specifically cytotoxic for target cells bearing the major histocompatibility antigen of the doner, on mixing, an interaction occurs which involves intimate binding of effector to target ending in death of latter cell. The degree of cytolysis can be assessed by measuring the release of radioactive chromium from prelabelled target cells [Roitt, 1977].

DIAGNOSIS OF DERMATOPHYTOSIS :

Since the diagnosis of ringworm infection by purely clinical means is not always possible, and since the identify of the causal fungus is important both epidemiologically and for purposes of treatment. Both direct and cultural examinations of selected material should be made whenever possible (Young et al., 1982).

I. Clinical Diagnosis :

Clinical manifestation of tinea or ring worm infections are so variable that diagnosis on this base alone is unreliable. The presenting signs and symptoms are often identical to those produced in a variety of skin diseases. The clinical appearance of any tinea is variable and is determined by the infecting organism, the site of infection, the patient's immune response and treatment (Nath and Agarwal , 1972).

The dermatophytic infections can be manifested as :

1. *Tinea Capitis* (Ringworm of scalp) :

Tinea capitis include dry, scaly lesion of the scalp stimulating alopacia areata because the hair are broken off, either below the scalp surface "Black dot ringworm" or 1-2 mm above the surface "greypatch", it also cause deep inflammatory boggy lesion known as "kerion" (Rippon, 1982).

2. *Tinea corporis* (Ringworm of the body, tinea circinata):

It is characterized by a circular lesion with varing degree of inflammation and maintain its circular pattern by a uniform rate of radial (centrifugal) growth of the dermatophytes at the periphery. The border is active with vesicles and raised crustes and tend to heal at the center [Emmons, 1970].

3. *Tinea pedis* (Foot ringworm, Athlete's foot) :

Infection of the feet or toes with a species of dermatophytes presents itself in several ways as itching, pain, foetid odour or it may be symptomless and only discovered during routine examination [Emmons, 1970].

4. *Tinea cruris* (Ringworm of the groin) :

It began as erythematous, scaling or vesicular patch that spread peripherally and clears at the center with well defined edge. It may be acute or chronic but usually severe pruritis develop [Domoukes, 1971].

5. *Tinea manum :* (dermatophytosis of the hand) :

It may take several different clinical forms, as hyperkeratosis of the palms and fingers (usually unilateral), crescentic exfoliating scales; circumscribed vesicular patches, papular and follicular scaly patches, Also erythematous scaly sheets on the dorsal surface of the hand [Nath and Agarwal, 1972].

6. *Tinea barbae* : (Ringworm of the beard) :

Ringworm of the beard and moustache areas of the face with invasion of coarse hairs, it is thus a disease of adult male. Tinea of chin and upper lip in females and children are considered as tinea faciei i.e. ringworm of the glabrous skin of the face. The animal species T. verrucosum and T. mentagrophytes are responsible for great majority of cases [De Vroey, 1985].

There are two clinical types-deep (nodular) and superficial (crusted) which caused partially bald patches with folliculitis. The superficial crusted type causes mild pustular lesion with broken off hair. The deep type produce a slowly and kerion-like swelling [Emmon et al., 1970].

7. *Onychomycosis* (Ringworm of the nails, Tinea unguium):

Nail that are deformed by pressure of the shoe or adjacent to infected skin are most frequently involved (Emmons, 1970). So, onychomycosis is more common in toe nails particularly big toe nail caused by narrow foot wear [Ramesh et al., 1983].

II. Laboratory diagnosis :

a) *Direct examination :*

It is the routinelly used, simple and inexpensive methods. In which the skin, hair or nails are removed from infected areas and placed onto a microscopic slide to which 1-2 drop of 20% KoH are added and cover slip is applied. Gentle heating or keep it at room temperature for (15-20) minutes to dissolve cellular materials leaving the hyphae and spore more easy identificable [Rippon, 1982].

Because nail tissue is very hard to obtian a good direct microscopic examination, it may be necessary to incubate the pieces of nail for half an hour in KoH 30% [Bulmer, 1983].

For all types of specimens, hyphae must be differentiated from other artifacts. These include fibres of cotton, wool, and synthetic materials, fat droplets, and "mosiac fungus". Mosiac is a network of materials including cholesterol crystals, which is deposited around the priphery of keratinized epidermal cells. It can be seen to follow the outline of the cell but not to go through it. This observation together with the lack of internal organells, differentiates it from true hyphae [Sinski, 1974].

b) *Culture Methods :*

Material from suspected cases of mycotic infection should be cultured, even though direct examination of the material is negative (Nielson, 1981). Because ringworm can appear quite variable and culture is such an easy procedure, it is advised as a routine part of dermatologic examination. Inoculation should be made with straight or hooked nichrom wire on a solid medium, inocula should be pressed into the surface. Several tubes and multiple inocula are recomended for each sample (Beare, 1972).

The standard media for primary isolation of dermatophytes is Sabouraud's dextrose agar (S.D.A.), containing cyclohexiмide (Actidione) in a concentration of 0.1 to 0.4 mg per ml to supresses the growth of most saprophytic fungi without affecting the growth of pathogenic dermatophytes. The various antibacterial antibiotics used include chloramphenicol(0.05mg per ml) or aureomycin (0.1 mg per ml); both are satisfactory. Growth is relatively slow; usually ten days to three weeks are required at the optimum tempratures (26-28°c). When the growth becomes evident, identification of dermatophytes require careful observation of gross colonial morphology and microscopic examination of properly prepared samples using lactophenol

cotton blue mounting. These prepared slides allow the visualization of spores, conidia arrangement, and mycelial appendages. During macroscopic examination of culture, the media used, date of inoculation, rapidity and luxuriance of growth should be noted. Microsporum gypseum grow in about 3-5 days, M. canis in about 5-10 days while T. violaceum grow about 15-20 days and T. schoenleini in about 30 days. The surface configuration of the colonial growth aids the diagnosis, as it may be rounded (M. gypseum), downy (M. audouinii), flat (T. persicolor), folded or cerebriform (T. schoenleinii & T. soudanense). The margin of colony may be sharply defined or may fade into the medium. The texture of colonial growth may be cottony (M. canis) glabrous (T. violaceum), powder (T. mentagrophytes) or woxy (T. schoenleinii and T. verrucosum). The colour of the colony and the medium is an important feature and aids identification of dermatophytes. A violet color of both colony and medium is characteristic of T. violaceum and a yellow - orange colony is seen in M. canis [Myrvik and Weiser, 1988].

During Microscopic examination of the colonial growth, the various vegetative and reproductive forms should be noticed [Delacretaz et al., 1976].

1. Vegetative Forms : [Hartmann & Rohde, 1980] (as in Fig. 1)

1. *Mycelium, hyphae and thallus :*

These terms indicate thread like sterile organic materials, which may be septated or non-septated or may be present in small or large masses.

2. *Pectinate bodies :*

Are mycellial formation, forming series of lateral bads in comb-tooth fashion. They are characteristic of M. oudouni, T. schoenleinii and T. mentagrophytes.

3. *Racquet mycelium :*

They can usually be seen in any species of microsporum, in E. floccosum and in T. mentagrophytes.

4. *Nodular organ :*

It is rounded, ball like structure formed from a mass of interwinded hyphae, it is seen in M. gypseum, T. mentagrophytes and in other species.

5. *Favic chandeliers :*

They resemable reindeer horns and are usually seen in T. schoenleinii.

6. *Spirals :*

Cork screw like turns of mycellium seen in older fluffy portions of T. mentagrophytes.

7. *Arthrospores :*

A modification of the hyphae with thickening of the walls in short segments. It may occures singly or in series.

8. *Chlamydospores :*

These are large, thick walled spores which observed along the course of hyphae at its terminus or on a lateral branch, particularly in T.schoenleini or candida albicans and E.floccosum.

II. Reproductive structures :

A sexual reproduction occur by conidia which are classified into macroconidia or as microconidia according to size.

Macroconidia : [Hartmann & Rohde, 1980] (as in Fig.2)

In trichophyton species they are poorly developed, thin walled, smooth, cylindrical with 4.6 transverse septae and are produced in few number.

In microsporum species the macroconidia are fusiform with 5-15 septae and their walls are thick and roughned with pits or tubercle like projection.

In Epidermophyton species the macroconidia are pyriform or oval, broadened and rounded at their distal poles with smooth thick walls and an overage of four septae. They usually occur in clusters.

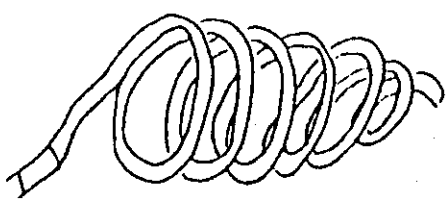
Pectinate organ

Central chlamydospores

Raquet mycelium

Nodular organ

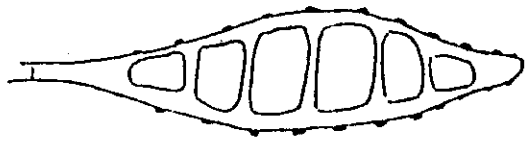
Terminal chlamydospores



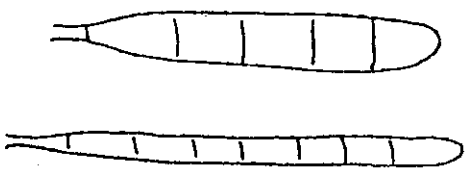
Spiral

Fig. (1) : Vegetative form

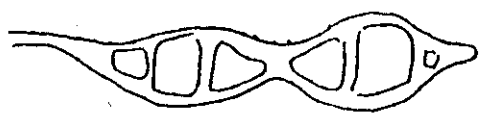
T. mentagrophytes



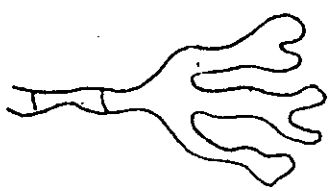
M. canis



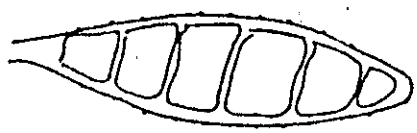
T. rubrum



M. audouini

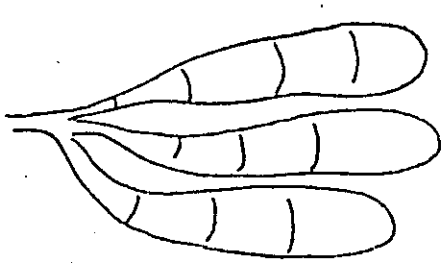


Favic chandeliers

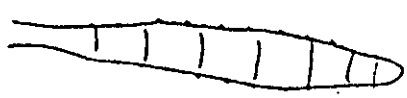


M. Gypseum

M. fulvum

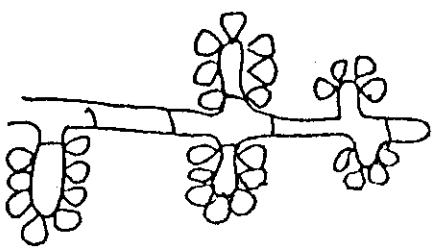


E. floccosum

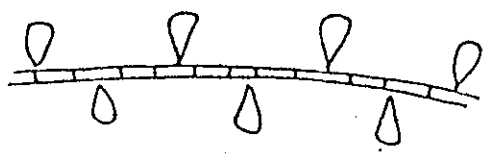


M. persicolor

Fig. (2) : Some macroconidia



T. mentagrophytes



T. rubrum

Fig. (3) : Some microconidia

Microconidia : Hartmann & Rohde, 1980] (as in Fig.3)

They are small, rounded or oval spores may occuring singly or in grape like clusters, they are present in most microsporum and some trichophyton species and may be absent in others as T.violaceum., T.verrucosum, T. schoenleinii and Epidermophyton [George et al., 1957].

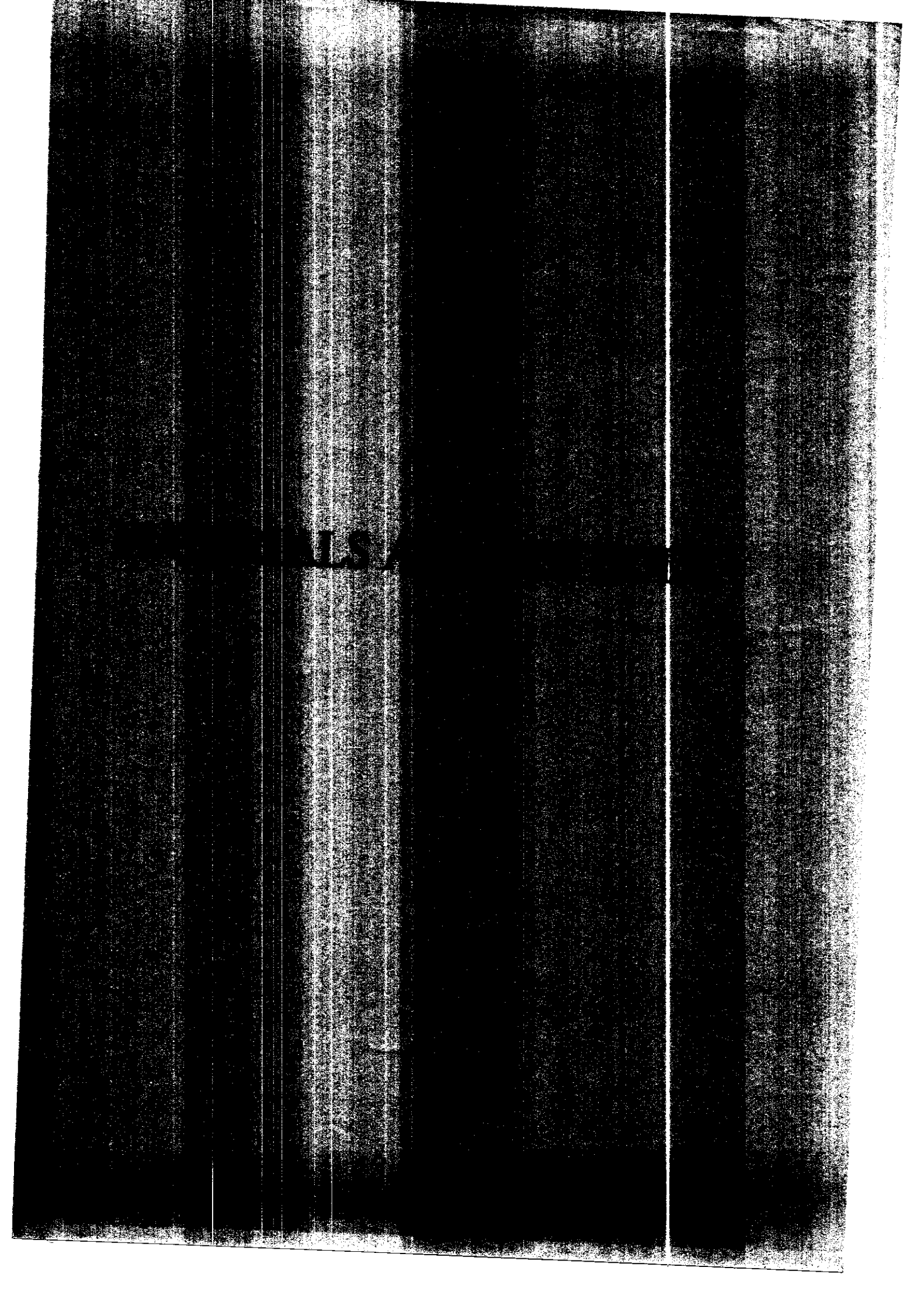
MANAGMENT OF DERMATOPHYTE INFECTIONS :

* Therapy :

The dermatophytosis are often self-limiting alternativelly they may persist for many years, patients with severe or disfiguring lesions require prolonged oral treatment with Griseofulvin or the alternative drug ketoconazole. Miconazole and clotrimazole are most effective agents for topical treatment. Since tinea pedis is made worse by shoes that confine perspiring feet, powders or other means of keeping the feet dry are sometimes helpful. Oitments containing tolnaftate or fatty acid salts may be effective for treating tenia of glabrous skin [Sveigaard, 1986; Myrvik and Weiser, 1988].

* Control of disease transmission :

The dermatophytosis are best prevented by avoiding exposure to causative fungi. In infections with M. audouinii, the organisms are transmitted from one child to another, especially by contact with caps or other heavily



MATERIAL AND METHODS

CASE MATERIALS :

Two groups were included in this study :

Group A : Consists of 60 patients (28) males and 32 females. Their ages ranged from 2-62 years with different types of dermatophyte infections as they were diagnosed clinically. 14 patients with tinea capitis, 15 with tinea circinata, 9 with tinea pedis, 7 with tinea cruris, 6 with tinea manuum, 3 with tinea barbae and 6 with onychomycosis.

These patients were attending the Dermatology and Venereology. Out Patient Clinic of Benha University Hospitals between the period from December 1991 to August 1992.

Group B : Control group consisted of (30) normal subjects completely free from any dermatological lesion. They were in the same range of age and sex as the case group. The patients and controls were subjected to thorough medical history to be sure that they were free from any immunodeficiency diseases and not under immunosuppressive drugs e.g. corticosteroid therapy or prolonged antibiotic therapy.

We have two main objectives for our study :

A) Laboratory diagnosis of dermatophytes infection.

B) Immunological study for testing T.cell function.

A) Laboratory diagnosis of dermatophytes infection :

a) *Sampling :*

Samples were taken according to [Roberts and Mackenzie, 1986] scrapings were taken from the definite edge of the lesion by a fairly blunt scalpel flamed prior to use, for the tinea circinata and tinea cruris. For tinea pedis, flamed epilation forceps was used to pull away the outer debris and macerated tissue from the sole and interdigital spaces. For tinea capitis, hair is plucked together with scraping of the scalp surface. Nails were cut back approximately as possible then the under surface of the junction of the nail with the nail bed was scraped. The specimens were collected direct on to a microscope slide held against the skin. This is covered with a second slide and the two then wrapped up in folded paper. The samples were labelled by the number of the patients.

b) *Direct microscopic examination :* [Rippon, 1982] :

Some of the collected material was soaked in few drops of 20% potassium hydroxide solution (KoH) on a slide and covered with a cover slip. The preparation was heated gently short of boiling as this precipitates KoH crystals. The slide was allowed to cool few minutes before examination. The potassium hydroxide clears the specimen (i.e. keratinized cells are rendered transparent so that the fungal filaments and

spores are easier to see) by digesting protienaceous debris, bleaching pigments and loosening the sclerotic materials without damaging the fungus. The preparation was then examined by the low and the high power of light microscope for the presence of hyphae and arthrospores.

c) *Culture of the specimens* [prevost, 1983] :

Fungal culture provides a definitive diagnosis and were performed in all patients suspected of having dermatophyte infections even in negative KoH preparation.

MEDIA USED FOR ISOLATION AND IDENTIFICATION OF DERMATOPHYTES :

1) **Sabouraud's glucose cycloheximide chloramphenicol agar** [Rippon, 1982]:

Ingredients of the medium :

Glucose	10.0 g.
Peptone	10.0 g.
Agar	15.5 g.
Cycloheximide	0.4 g.
Chloramphenicol	0.05 g.
Distilled water	1000 g.

pH : 5.8 - 6.2

Addition of cycloheximide (Actidione) to the medium suppress the growth of most saprophytic fungi and

chloramphenicol suppress the growth of bacteria. The medium was sterilized in the autoclave at 121° for 15 minutes.

2) Dermatosel agar (Dean & Haley, 1962) :

Ingredients of the medium :

Dextrose	20.0 g.
Mycological peptone	10.0 g.
Agar	14.5 g.
Distilled water	1000 ml.

pH 5.8 - 6.2

We added 2 vials of antibiotic supplement re-consistituted with 6 ml acetone, cyclohexiimide 0.4 g/L and chloramphenicol 0.05 g/L., then they were mixed gentely and sterilized by autoclaving at 115°C for 15 minutes.

For selective separation of dermatophytes vials of commercially available dermatophytes test medium were used (biomerieux). This medium developed by Taplin et al., (1969). It contains cyclohexiimide to suppress contaminant fungi. Chlortetracycline and Gentamycin to suppress bacteria and phenol red as an indicator that turns the medium from yellow to red at the alkaline pH that results from dermatophytes growth.

Ingredients of Dermatophyte Test Medium (DTM) :

Phytone	10 gm.
Dextrose	10 gm.
Agar agar	20 gm.
Phenol red	1 ml.
Cyclohexi mide	0.5 gm.
Gentamycin sulphate	100 µ/ml.
Chlortetracycline Hcl	100 µ/ml.
Distilled water	1000 ml

pH 5.5.

IDENTIFICATION OF THE FUNGAL GROWTH :

Dermatophytes growth was identified according to Conant, (1971) as follows :

1. *Macroscopic examination of the colonies :*

This constitutes a fundamental criterion in the identification of the dermatophyte colonies. The texture, appearance, colour of the surface were noticed.

2. **Microscopic examination of suspected growth :**
Direct examination of stained films :

A portion of the colony was removed by stiff-wire needle and placed in a drop of lactophyenol cotton blue on a slide and teased apart with two sterile needles.

The preparation was then covered with a cover slip and examined under the low and high power of the microscope.

Lactophenol cotton blue mounting fluid [Rippon, 1982] was used as a fluid mount between slide and cover slip, for examining the fungal preparations, it was composed of :

Lactic acid	20 g.
Glycerine	40 g.
Distelled water	20 ml.
Phenol crystals	20 gm (melted in water bath before weighing).
Cotton blue (or methylene blue)	0.05 g.

Preparation :

Lactic acid, glycerine and distilled water were mixed with melted phenol and dissolved with even heat. Cotton blue added to the mixture and dissolved. Filteration is done through filter paper and then placed in dropping bottles.

The following structures was important for identification of Dermatophytes in examination of the stained films with lactophenol cotton blue :

Microconidia :

Small, single called spores, oval, rounded or pear shaped, borne along the side of the mycelium or terminally in grape like structures.

Macroconidia :

Large septate spores which vary in size, form and character of their walls.

Chlamydospores : Thick walled spores, intercalary or terminal.

Arthrospores :

A sexual spores formed by fragmentation of the mycelium.

B) **Immunological study for testing T-cell function of patients with dermatophyte infections :**

Blood samples were taken from the patients and control group on heparin free of preservative under a septic precaution to separate lymphocytes which were tested for :

1. Numerical evaluations of T.lymphocytes using E.rosette technique.
2. Lymphocyte function by blastoid transformation assays, using phytohaemagglutinin (PHA) mitogen.

1. E.ROSETTE TEST :

T. cells were counted by E. Rosette methods [Hudson & Hay, 1976].

METHODS :

A. Isolation of lymphocyts from whole blood :

Lymphocytes were separated from peripheral venous blood by Ficol/Hypaque gradient technique [Follwing the methods of Boyum (1966).

This method need :

a. Preservative free heparin (Sigma Chemical Co.).
b. Hanks balanced salt solution (Flow Laboratories).
c. Ficoll/Hypaque solution (Sigma Chemical Co.).
d. Sterile pasteure pipettes (Biomeriex).
e. Sterile centrifuge tubes.

PROCEDURE :

1. 5 ml freshly heparinized blood were diluted with equal volume of Hanks solution.
2. Carefully, we overlying 8 ml. diluted blood on 4 ml. Ficoll/Hypaque gradient without allowing the solution to become mixed, by keeping the pipette against the tube wall 5-10 mm. above the fluid mensicus. Centrifugation at 2000 rotation per minute (r.p.m.) for 20 minutes, at room temperature, the lymphocytes would be localized as a whitish layer on the upper meniscus of gradient solution. Using fine pasteur pipette. We

take up the zone containing the lymphocytes and washed twice in Hanks solution (10 minutes at 1200 r.p.m.).

3. Viable lymphocytes were tested with trypan exclusion test and adjusted at concentration to 2 x 10^6/ml. in hanks solution.

Trypan blue exclusion test (Boyse et al., 1964) was used to determine the number of viable cells in the original suspention. The cells to be counted are gently suspended in the medium, and 0.1 ml of the suspension was added to 0.1 ml of 0.16% solution of trypan blue (Sigma) in isotonic saline. The suspension was then incubated for 10 minutes at 37°C and the number of viable cells counted in a haemacytometer within 5 min. after incubation.

B. Preparation of sheep red blood cells :

Sheep blood cells were collected asptically in equal volumes of sterile Alsverser's solution the cells were allowed to age at 4°C for one week before use. In 10 ml graduated centrifugue tube, the blood was centrifuged at 1000 r.p.m. for 5 minutes at room temperature, the supernatant was removed carefully by suction and discarded. the tube was then filled to the top mark with Hank's Solution and the packed cells were gently resuspended by a pasteur pipette and centrifuged, the washing was repeated twice. If hemolysis was observed, the blood was discarded. A 1% suspension of sheep red

cells was prepared by mixing 0.02 ml of packed cells with 2 ml Hank's Solution.

C. Rosetting technique (According to Hudson & Hay, 1976):

1. 0.1 of lymphocytes suspension was pipetted into a Wasserman tube and 0.1 ml SRBCs was added two replicates was done.
2. The lymphocytes were mixed well with SRBCs by centrifigation at 1000 r.p.m. for 4 minutes at 4°C.
3. Without resuspending the deposited cells were incubated in ice overnight.

D. Counting rosettes :

The cells were gently resuspended and lymphocyte were inspected for a rosette formation in a haemocytometer. A rosette forming cell (RFC) was defined as lymphocyte which had three or more SRBCs adhere to its surface, the number of RFC was expressed per 100 lymphocytes.

2. LYMPHOCYTE BLAST TRANSFORMATION (Stites, 1987) :

REQUIREMENTS :

This test needs :

1. Heparinized blood, free of preservative (Sigma Chemical).
2. Hanks balanced salt solution (Flow Laboratories).

3. Ficoll/Hypaque solition (Flow Laboratories).
4. 20% Fetal calf serum (Flow Laboratories).
5. Phyto heamagglutinin (PHA) (Difico).
6. R.P.M.I. 1640 medium (Roswell Park Memorial Institute medium (Gibico).
7. Sterile 96 rounded bottom tissue culture plates.
8. Giemsa stain.

METHODS :

1. Separation of viable lymphocytes on Ficoll/Hypaque gradient as described previously.
2. The lymphocytes were a septically washed twice and the concentration was adjusted to 2×10^6 ml. in PRMI 1640 media, supplemented with 600 μ/ml. pencillin, 0.1 mg. streptomycin, 1% (L) glutamine, 25 μm Hepes buffer and 20% fetal calf serum.
3. The lymphocyte were plated in triplicated in steril 96 rounded bottom tissue culture plates.
4. Phytohemagglutinin (PHA) was used in varying concentration 5.0 μg/culture, 2.5 μg/ culture and 1.25 μg/ culture, added to the lymphocytes.
5. Cell culture were incubated at 37°C in 5% Co_2 atmosphere for 72 hours, at which time mitogen produce their maximum effect on DNA synthesis.
6. After culture, many films were stained by Giemsa and average percentage blast were made.

STATISTICAL ANALYSIS

Statistical analysis of the results were carried out as the following formula : (Pipkin, 1984).

Mean Value :

$$\bar{X} = \frac{X}{n}$$

where : X = Sum of observation.

n = number of observation.

Standard Deviation (S.D.) :

$$S.D. = \sqrt{\frac{(X - \bar{X})^2}{n - 1}}$$

Where $(X - \bar{X})^2$ = sum of square of the difference between each observation (X) and mean value all observation ($\bar{X}$).

T-test :

The best of significance between 2 means :

$$T = \frac{M_1 - M_2}{\sqrt{\frac{(S.D_1)^2}{n_1} + \frac{(S.D_2)^2}{n_2}}}$$

where : M_1 = mean value of sample 1.

M_2 = mean value of sample 2.

n_1 = number of cases in sample 1.

n_2 = number of cases in sample 2.

$S.D_1$ = Standard Deviation of sample 1.

$S.D_2$ = Standard Deviation of sample 2.

The probability (P) for this calculated value of T. with degree of freedom.

$P = (n_1 + n_2) - 2$ was obtained from statistical table.

The level of significance accepted for P is < 0.05