THE TOXICITY OF MYCOBACTERIUM ULCERANS

BY AMIN A SHARIF

Department of Medical Microbiology University College
and Middlesex School of Medicine School of Pathology
Ridinghouse Street London W1P 7PN

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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ABSTRACT

The mycobacterial genus is responsible for two of the most devastating diseases of man, namely tuberculosis and leprosy. In addition to these two classical diseases, there are a number of specific and non-specific infections due to mycobacteria. Such infections are rare compared to tuberculosis and leprosy, but they may cause crippling or even fatal diseases. Mycobacterium ulcerans is unique amongst mycobacteria in its tissue necrosis and local immunosuppressive effect at the site of the infection. This has been attributed to a toxin about which little is known. In this project I examined 31 strains of M. ulcerans isolated from three different continents for:

1. Pathogenicity in mouse foot-pad, which usually results in progressive swelling, except that three strains used in this project failed to do so. This observation led me to put forward the theory that the non-pathogenic form is present in the environment. The reservoir of the organism has never been found in the environment, perhaps because the work by which the organism is usually recognised is mouse pathogenicity, without which it could easily be missed. The pathogenic strains would only have to be present in small numbers to give rise to the sporadic human infections that occur.

2. The pathogenic and non-pathogenic strains were subjected to a wide range of identification methods to prove their similarities.

3. The culture filtrate of both pathogenic and non-pathogenic strains were examined in lymphocyte transformation tests (LTT) to establish whether there is a link between the suppressive effect on lymphocytes and loss of pathogenicity.

4. Attempts to isolate the suppressive factor have been carried out using HPLC and I have examined the possibilities that this suppressive factor may be phage or plasmid mediated.

5. Since BCG immunisation and anti-mycobacterial chemotherapy has proven to be ineffective in cases of M. ulcerans infection, I looked for the possibility of preventing, or delaying, the onset of the disease by vaccinating with non-virulent strains and culture filtrates.

LIST OF TABLES, PHOTOGRAPHS AND GRAPHS

CHAPTER ONE

BRIEF CHARACTERISATION OF BACTERIAL TOXINS

The ability of any bacterial species to colonise tissues depends on a number of interrelated properties. These include whether the bacteria can:

a) gain access to one of the body surfaces of the host;
b) overcome the non-specific mechanisms and establish itself on the surface;
c) obtain suitable nutrients from the micro-environment to multiply in the host.

Most bacteria can gain access to the body through the gaps leading to the interior, but some penetrate the skin surface as a result of wounds. No matter how the organism enters the body, the outcome of a primary bacterial challenge is either clearance of bacteria or appearance of clinical signs of disease.

The concept of harmful poisons being formed as a part of an infectious disease was demonstrated at a very early stage of bacteriology. The observations of Klebs, Koch, Loeffler, Roux and Yersin introduced the foundations for the role of bacterial toxins as virulence determinants in disease. The criteria for judging whether a toxin is responsible for the clinical signs of disease were summarised by Heyningen, 1955.

They include:

a) the organism is known to produce a toxin;

b) virulent variants produce the toxin and avirulent strains do not;

c) injection of the toxin separately from the bacteria produces symptoms that mimic the disease;

d) the disease can be prevented by immunisation against the toxin.

There are problems with these criteria, mainly the difficulties of applying these specifications to bacterial species where the significance of toxins in natural disease has not been established.

BACTERIAL ENDO-EXOTOXINS

One of the earliest and most useful distinctions in the classification of bacterial toxins was the differentiation between endotoxins and exotoxins. Endotoxins are structural components released from the walls of dead bacteria after autolysis, whereas exotoxins are soluble substances released by growing bacteria.

ENDOTOXINS

Endotoxin was first recognised by Pfeiffer (1892) in cultures of Vibrio cholerae. It is a heat-stable component that causes a wide range of pathological effects in susceptible hosts. It is differentiated from the exotoxin produced by actively growing cultures of this organism. Later it became clear that all species of Gram negative bacteria produce endotoxins as part of their cell wall. The studies characterising endotoxins led to our current understanding of the subject. Lipopolysaccharide (LPS) consists of lipid A, a core oligosaccharide and cell surface O-antigen (Boivin and Mesrobeau, 1935; Westphal, Gineiner, Tanaka, Eichenberger, 1969; Westphal and Luderitz 1954). LPS plays an important role in stabilisation of the Gram negative cell wall, serves as a receptor for bacteriophages and forms the basis for a serological classification which is a vital aid in the diagnosis of infection. Purification and hydrolysis of LPS indicated that lipid A is the active principle (Westphal *et al*, 1969). It has been suggested that endotoxic properties of pyrogenicity and lethal toxicity are due to accumulation of NADH and ADP, leading to enhanced glucolytic metabolism and lactate formation resulting in accumulation of superoxide radicals and hydrogen peroxide (Bradley *et al*, 1979). LPS is a T-independent mitogen that produces polyclonal B-cell activation and a predominantly IgM response and stimulates macrophages (Morrison, 1983). Endotoxin is a very potent trigger for tumour necrosis factor (TNF) release, by which part of the tissue damage is mediated. (Rook et al 1986)

BACTERIAL PROTEIN TOXINS (EXOTOXINS)

During active stages of growth, bacterial species produce soluble proteins. These products differ in a number of ways from endotoxins. They are considerably more toxic than endotoxins (Gill, 1982). Exotoxins can often be converted to toxoid, which stimulate the production of antibodies which usually neutralise the effect of the toxin. Exotoxins can be divided into three groups:

a) cytolytic toxins;
b) toxins with intracellular activities;
c) toxins whose mechanism of action and molecular structure are not fully understood.

CYTOLYTIC TOXINS

This type of toxin can be detected by the ability of bacteria such as Clostridium novyi, type D to cause considerable damage to erythrocytes on blood agar plates (Bernheimer, 1970) and has been termed cytolysin (Bernheimer, 1970). Cytolysins are produced by a wide range of Gram positive and Gram negative bacteria. Many of these cytolysins can cause death when injected in sufficient amounts into laboratory animals. Some are capable of introducing permeability changes without causing cell lysis (Gill, 1982; Thelestam and Molby; 1975 Wilkinson, 1975). Cytolytic toxins are divided into phospholipases, thiol-

activated cytolysins and cytotoxins that can affect leukocytes. Phospholipases are produced by many pathogenic and non-pathogenic bacterial species designated A to D, and these hydrolyse glycerophospholipids and sphingolipids, which are the two major classes of phospholipids in mammalian cell membranes (Mollby, 1978). C. perfringens types A, B, C and D produce a phospholipase termed α toxin. Type A strain produces the largest amount of this toxin which is associated with massive destruction of tissue accompanied by severe toxaemia and shock (Bullen, 1970). An unusual phospholipase has been identified as the lethal toxin of Corynbacterium pseudotuberculosis, the cause of suppurative infections mainly in sheep, goats and horses. The toxin is a basic glycoprotein. In natural infection there is no evidence of toxicity, and haemolytic activities and cellular permeability are absent, but it causes platelet aggregation and releases vasoactive substances which contribute to the spread of the infection (Mollby, 1978). S. aureus produces a wide range of extra-cellular products including exfoliative toxins which include α toxin, β toxin, γ toxin, Δ toxin and leucocidin. The toxins have always been considered to be most significant in the pathogenicity of staphylococcal infections (Mollby, 1978; Retter, 1985). Thiol-active cytolysins are produced by a wide range of Gram positive bacteria. They are sulfhydryl-activated cytolysins, otherwise known as oxygen labile haemolysins, which bind to membranes and lead to cell lysis (Smyth and Duncan, 1978). Streptolysin O is one of the best known thiol-active cytolysins produced by β-haemolytic streptococci isolated from humans.

In addition to streptolysin O, groups A, C and G streptococci produce streptolysin S which cause suppression of T-cell dependent antibody responses and helper T cell function (Wannamaker, 1983).

TOXINS WITH INTRACELLULAR ACTIVITIES

There are a large group of toxins which affect metabolic activities of cells following binding to receptors on the plasma membrane and transportation across cell membranes. The main examples are cholera and pertussis toxins which activate adenylate cyclase, and diphtheria toxin and pseudomonas exotoxin A which inhibit protein synthesis inside the cell (Eidels *et al*, 1983; Middlebrook and Doreland, 1984). The common feature of these toxins is a polypeptide chain which can be cleaved by proteolytic enzymes into two chains linked by disulfide bonds (eg. diphtheria toxin); or the polypeptide chains are synthesised separately and then associated to form two chain structures (eg. cholera toxin). The B chain is the larger of the two and binds the toxin on to the cell enabling the molecule to enter the cell (Stephen and Pietrowski, 1981).

There are other important toxins whose structures have been well characterised but the intracellular activities are less well defined. These include tetanus and botulinum toxins. Although the neurotoxin of C. tetani is well characterised, there is still no *in vitro* assay for it (Bizzin, 1979). Several studies have suggested that tetanus toxin

might use the receptor system of thyroid-stimulating hormone, but the relevance of this observation to its *in vivo* activities is not clear (Eidels *et al*, 1983).

TOXINS WITH UNCHARACTERISED BIOCHEMICAL ACTIVITIES

There are a number of toxins where there is good evidence that they are the major determinants in the pathogenesis of disease but little is known of their biochemical activities or whether they have the A-B structure. They include anthrax toxin and the β and ϵ toxins of C. perfringens (Sakurai *et al*, 1978). Massive septicaemia is a prominent feature of the terminal stages of anthrax, but the significance of toxaemia in the pathogenesis of anthrax is not clear (Lincoln *et al*, 1970; Leppla, 1982).

TOXICITY IN MYCOBACTERIA

My search in the literature for evidence of toxicity in Mycobacteria concluded that the only toxic substance extracted from many different species, not all naturally produced, is the cored factor. Further discussion of this matter follows in Chapter Seven.

M. ulcerans is the only species in the genus of Mycobacterium which is known to the mycobacteriologist to produce a soluble toxin during the course of the disease. This is based on evidence obtained from histological studies of the infection, yet this toxin has not been identified or isolated, despite its apparent importance in the pathology of the disease. This project has been carried out to develop further studies of M.ulcerans and its toxicity.

CHAPTER TWO

HISTORICAL INTRODUCTION TO MYCOBACTERIA

The mycobacterial genus is responsible for two of the most devastating diseases in the recorded history of civilisation, namely tuberculosis and leprosy.

Tuberculosis has been referred to as the king of disease in an ancient Hindu text, and called by John Bunyan "captain of all these men of death" (Crowford P, 1911).

Although in the past tuberculosis was often confused with other diseases, its basic nature, lasting effect and destructive character has always been recognised (Waksman, 1964). Hippocrates (460 - 377 BC) the father of medicine, described in detail the clinical features of pulmonary and spinal tuberculosis and applied the name "phthisis" which meant both to melt and to waste the body (Calmette 1923; Burke 1955; Bankoff 1946). Leprosy on the other hand became known as the disease of kings when Robert Bruce, King of Scotland, numbered as one of its victims (Crowford P, 1911). The horrifying reputation of leprosy developed from its ability to cause terrible disfigurement and crippling. Early writings by the Indians and Chinese have described the features of leprosy. Throughout history until the present day, leprosy victims have been socially outcast which led to their poverty and distress. In places called Lazar houses or leprosaria, leprosy patients were isolated, and some churches had what are called hagioscopes, small openings in the wall through which the leprosy

victim could observe religious services (Brothwell D, 1967; Keers, 1978). Until the early part of this century, cases of leprosy occurred in Scandinavia and cases still occur in Southern Europe, and are common in other parts of the world like South America and India.

Mycobacteria have a large nomenclature connected with them, especially tuberculosis. "Scrofula", "strumon" and "the king's evil" are names given to tuberculous glands of the neck. Another Latin name, "gibbus", is used to describe tuberculosis of the spine (Bulloch W, 1930) and the names of famous medical figures have been attached to some of its manifestations, such as Pott's disease of the spine, Pott's puffy tumour and Ghon's focus.

First attempts to classify and name mycobacteria were made at the end of the last century, when there was a general interest in speciation of the newly discovered bacteria. "The Atlas of Bacteriology" (1896) by Lehmann and Neuman recorded many of these efforts.

The genus Mycobacterium contained two species initially; Mycobacterium tuberculosis observed by Robert Koch in 1882 and Mycobacterium leprae observed by Armauer Hansen in 1874. The name Mycobacterium was taken from the characteristic feature of the bacilli. When grown on liquid media they appear as "fungus-like bacteria", although an earlier name for them - proactinomycetacaea - also reflected this, but has been relegated because of the general acceptance of Mycobacterium (Burke, 1955; Keers, 1978).

The leprosy bacillus has never been grown *in vitro* with complete certainty, but it was included in the genus because of the acid fast property shared with tubercle bacilli (Burke, 1955). Although the leprosy bacillus was described first, it was not selected as the type species of the genus because of its lack of culturability. Later, more and more bacilli were added to the genus, which were isolated from several environmental and clinical sources, and found to have the same cultural and staining characteristics. At first considered to be varieties of the tubercle bacillus, they soon became accepted as distinct species in their own right, although the continued use of the term "atypical" mycobacteria still reflects this old misconception. In addition to the two classical mycobacterial diseases, there are a number of specific and non-specific infections due to mycobacteria. Such infections are rare compared to tuberculosis and leprosy, but they may cause serious crippling or even fatal diseases and have reached special prominance since the development of the AIDS epidemic.

Mycobacterium ulcerans was first described as the cause of disease in man, in patients from the Bairnsdale district in South Eastern Australia (MacCallum, 1948). Although there is evidence that the disease had been recognised by Sir Albert Cook in Africa in 1897 (Meyers *et al*, 1974). MacCallum and his colleagues did not propose a name for this new mycobacterium, which they called Bairnsdale bacillus, until 1950 when the name Mycobacterium ulcerans was suggested (Fenner, 1950). Since then, endemic areas have been found in Zaire, Uganda, Nigeria, Ghana, Cameroon, Malaysia, New Guinea, French Guiana, Mexico

and in different areas in Australia (John Hayman, 1984). Other local names were given to this infection such as Buruli ulcer, after an endemic area in Uganda called Buruli district. In Zaire, the disease is called "Mputa-Matadi" which means "itching stone", after a characteristic hard itching lump which develops during the early stages of the infection (J M Grange, 1980).

The history of mycobacteria has three important events. The first occurred at a meeting of the Berlin Physiological Society. The date was 24 March 1881. Robert Koch announced the isolation and culture of the causative agent of tuberculosis (Nocard 1895; Legge and Session 1898; Keers 1978; Collins and Grange 1983). Koch did not name his bacilli but termed them "tuberkelbaccilli" (bacilli of tuberculosis).

The second breakthrough in mycobacterial history was announced in 1891 as a result of Koch's studies on guinea pigs. Koch inoculated bacilli into the flanks of guinea pigs, and after a week he observed small firm nodules at the injection site, which later ulcerated (as explained below, this is remarkably similar to early events in M. ulcerans infection). Superficial inoculation into the opposite flank a month later produced a different lesion. Two days after the second injection, the site became black, necrosed and soon healed. This became known later as the "Koch phenomenon" or type IV hypersensitivity reaction. Further explanation of the Koch phenomenon will be mentioned later in this chapter (Nocard 1895; Brown 1941; Sigurdsson 1945; Grange and Bishop 1982). This immunological phenomenon has proved to be of

great value in diagnosis but has had far less influence in treatment than was hoped.

The other most important discovery in tuberculosis history was in 1944 when the first effective chemotherapy was found. Long before this time, people had been looking for a cure for mycobacteria diseases, especially tuberculosis and leprosy.

The first man to prescribe a remedy for tuberculosis was the Roman, Celsus (25 - 45 AD). He was aware of the necessity of early treatment in tuberculosis (Clamete 1923: Brown 1941; Bankoff 1946). He insisted that pulmonary ulceration was curable only in its early stages and regarded the earliest case as that seen after initial haemoptysis and before the onset of cough (Brown 1941). In 1546, Fracastorius spoke of tuberculosis as being a contagious disease due to an invisible germ in the lung (Webb 1936; Brown 1941; Bankoff 1946; Burke 1955).

Avicenna, the Arab physician (980 - 1037 AD) in his book (Al-Canon of Medicine) considered tuberculous ulceration had three stages, and was the first to employ intratracheal injection of infusion of red roses and honey as a favourite remedy (Burke 1955; Callmette 1923; Brown 1941).

When streptomycin was discovered as the first effective therapeutic agent against tuberculosis, fears of mycobacterial diseases were removed, and this was even stronger when discoveries of other anti-mycobacterial drugs followed (Burke 1955).

A considerable effort has been made by several bodies working in the field of mycobacteria to develop an effective vaccine and effective chemo-prophylaxis. Together these two factors would help to solve the problem of mycobacterial diseases world-wide.

Chemotherapy provides an effective measure against both tuberculosis and leprosy, but only when compliance with treatment is maintained and the level of drug resistance kept down.

Indeed, mycobacterial diseases have been virtually eliminated in countries were money and administration are available, but this is not true of countries where the facilities are unavailable, and large scale human suffering caused by tuberculosis and leprosy is still present.

Some 70 years ago, the bacillus of Calmette and Guerin (BCG), was developed as a vaccine against tuberculosis (Moore, 1981, 1983) and great enthusiasm was built up for this vaccine when tuberculosis was a major killing disease in much of the world. In 1933 some confusion in preparation of the vaccine led to the death from tuberculosis of 72 children in the town of Lubeck in Holstein. Reasonably, this led to questions about the safety and efficacy of BCG, and the setting up of controlled trials. The British Medical Research Council ran trials of BCG in UK against tuberculosis (British MRC Tuberculosis vaccines trials committee, 1963) and in Uganda against leprosy (Brown *et al* 1968). Both produced protection in the region of 80%, suggesting that BCG was an excellent vaccine for both diseases.

Subsequently, the World health Organisation carried out major trials against leprosy in Burma (Bechelli *et al*, 1974) and against tuberculosis in South India (Tuberculosis prevention trial, Madras 1979). The results were a disaster. Less than 20% protection was found in Burma and no protection was found in South India.

(KOCH PHENOMENON) Type IV Hypersensitivity Reaction

The original observation of Koch's phenomenon, described earlier in this chapter, was subsequently confirmed, and other infectious agents and antigens were shown to have a similar effect, such as Leishmania tropica. A general definition of the events which occur in this phenomenon can be described as: "The primary exposure to antigen or infection sensitises the animal, which develops a measurable degree of immunity. Subsequently, challenge with the same antigen produces the delayed type hypersensitive response, which is detected by the skin test." In 1945, it was found that this type of hypersensitivity is transferable by cells, but not by serum (Lansteiner and Chase, 1942). These two characteristics constitute the type IV hypersensitivity reaction (DTH).

There is evidence in the literature that proposes more that one response of this type. A non-necrotic response develops after a few days of challenging mice with Listeria monocytogenes (MacKaness 1962). In other studies, both necrotic and non-necrotic responses were

observed in guinea pigs challenged with tubercle bacilli (Debre and Donnet, 1922). Perhaps the most significant study which threw considerable light on the different mechanisms occurring in the tuberculin reaction was Wilson and Maier, 1940. After immunising guinea pigs with heat killed M. tuberculosis and challenging with living organisms, three groups of reaction were observed by the authors. These were:

a) Moderate response at the time of infection is favourable;
b) High response at the time of infection is unfavourable;
c) A very high response at the time of infection is worse than no response at all.

These three important conclusions highlight the immunological spectrum in this phenomenon and contain some important aspects of immunisation against mycobacteria.

Different mycobacterial species were found to evoke the different kinds of response. Studies of foot-pad tests with tuberculin-type reagents at different times after mice were challenged with different mycobacterial species (Rook and Stanford, 1979) demonstrated three kinds of hypersensitivity reaction.

1. Very early response follows challenge with all kinds of mycobacteria 6-7 days after immunisation and peaks 13-15 hours after foot-pad challenge. This appears to be similar to the response described by Jones and Mote (1934) in man.

2. Another type mainly caused by fast growing mycobacteria such as M. vaccae and by Glaxo BCG occurs 10-22 days after immunisation and peaks 18-23 hours after foot-pad challenge. This was thought to be similar to the response evoked by Listeria (Mackaness, 1962), and seems to occur as a mouse overcomes a challenge with a non-pathogenic species.

3. The late Koch-like response was found to be evoked by slow growing pathogenic mycobacteria such as M. tuberculosis and M. kansasii which appears 4-6 weeks after immunisation and peaks 40-48 hours after foot pad challenge. This appears to be the basis of immunopathology, and is the mechanism by which the mouse slows down the progress of inevitable disease, but does not overcome it.

It was found that these three responses in mice are transferable by cells from the draining lymph node. Moreover, it was noted that the response evoked by a particular organism can be changed by pre-immunizing mice with an organism predisposed towards the other type of response, ie if mice were immunised with a dose of M. vaccae and Glaxo BCG then challenged with M. kansasii, the Koch-like response does not develop (Stanford et al, 1980) and the animal does not succumb to M. kansasii infection.

More specific evidence was found supporting the different immunological DTH responses caused by various microbial agents and antigens. Miller *et al* (1975) reported transferring a delayed hypersensitivity reaction by T-cells responding to a specific antigen to unsensitised recipients. Subsequent challenge with the same antigen caused the same DTH reaction only if the recipient and donor T-cells express the same lymphocyte surface antigen coded for by the gene in the I region of the H-2 locus.

The involvement of the genes in DTH was also shown by the work of Zinkernagel, 1976. He showed that the transfer of DTH response to lymphocytic choriomeningitis virus (LCMV) from sensitised donors to a normal recipient was only possible when both donor and recipient T-cells express the same surface antigen coded not only by the gene in the I region of the H-2 locus, but also by the K or D region of the H^2 complex. He also noted (Zinkernagel *et al*, 1977) that transferable protective immunity to listeria in mice was under genetic restriction. Thus it is apparent that there is a relationship between DTH and involvement of H-C in mice, which may be involved in immunity against mycobacterial disease. The possibility of genetic susceptibility of humans to infection by leprosy and tubercule bacilli has led to the search for an association between genetic markers, in particular the MHC region and leprosy patients. In a controlled study directed at these markers, a marginal association between them and different leprosy populations has been found (Fine *et al*). The response to different types of mycobacterial antigens and the genetics of the individual undoubtably play an important part in both protective immunity and immunopathology.

Beside these two factors, the epidemiological evidence from skin testing of different populations support the idea of different responses of DTH. A range of different skin test reagents has been used in separate populations and simultaneously bacteriological samples from the environment of these populations have been examined. A relationship between the responses of the populations to the different antigens shows some correlation with the different species of mycobacteria isolated from that area (Paul *et al*, 1975; Stanford *et al*, 1973). In these populations, sensitisation is determined by the total amount of environmental exposure to mycobacteria and the species of mycobacteria present in this particular environment. In other skin test studies of populations, two different DTH responses were seen (Stanford *et al*, 1978). One form is characterised by oedema, erythema and no necrosis, and is thought to be associated with protective immunity. The other type is seen in patients with tuberculosis and leprosy and appears to be the Koch-like response. These epidemiological findings can be used to explain the failure of BCG Trials in Burma and South India, (Bechelli *et al*, 1974, Tuberculosis protection trials, 1974). In areas such as South India and Burma, a high degree of sensitisation by environmentally slow growing mycobacteria such as M. scrofulaceum or M. intracellulane leads to the development of Koch-like responses, abrogating the effect of subsequent vaccination with BCG (Stanford *et al*, 1978). It is anticipated that a non-necrotic delayed skin response is likely to be related to the best protection. Such protection is acquired following sensitisation by certain mycobacterial antigens (Stanford *et al*, 1980).

CHAPTER THREE

MYCOBACTERIAL SKIN INFECTIONS

Infection of the skin with mycobacterial species, including M. ulcerans, usually occurs following trauma or direct penetration. However, other routes of infection to the skin with mycobacterial species other than M. ulcerans are well documented.

It is not unusual for M. tuberculosis to cause skin disease by an exogenous route of infection. In Hong Kong, there were several cases where legs and buttocks of children developed lesions caused by M. tuberculosis (Wong, 1968). Also in India, the foot was a common site for the infection (Pandhi, 1977). It is presumed that such cases were caused by expectorated tuberculosis sputum. The lesion is a small papule which enlarges slowly over a number of months to form a warty plaque. This is often not inflamed and central healing can take place (Wilkinson, 1979; Pereira, 1976).

Although M. marinum is a pathogen of fishes (Aronson, 1962), occasional infection in humans can take place and this is by direct contact with the organism. Most cases recognised as M. marinum infection occur in those who are associated with leisure activities involving water, or fishermen, boating enthusiasts and keepers of tropical fish, (Linell, 1954; Janner, 1983). The infection in these lesions is recognised as typical mycobacterial infection in the presence of cell mediated immunity (Marsch, 1978).

M. chelonei is another opportunistic pathogen for both man and animal. Mainly, the infection is caused by direct introduction of the organism into the skin by contaminated materials. A series of accounts involving infection with M. chelonei have been reported from different parts of the world; most related to injection of contaminated solutions. Examples include the use of a histamine solution (Inman *et al*, 1969) and a batch of vaccines (Borghans *et al*, 1973). This species has two subspecies; the type subspecies and subspecies abscessus. They have different geographical distributions. The abscessus subspecies occurs in Africa and America and the type subspecies is commonly found in Europe (Stanford *et al*, 1977).

Another mycobacterial species associated with injection abscesses is M. fortuitum, but infection with this species is usually single and there is no recorded outbreak like those sometimes associated with M. chelonei (Cruz, 1938; Beck, 1965; Wells, 1955). M. fortuitum has also been found to have two subspecies; the type species and sub species giae (often called peregrinum (Grange, 1974)). The species has been classified into four serotypes using immunodiffusion analysis (Grange, 1973); serotype I is the type subspecies and serotype II, III and IV are the giae subspecies. Mycobacterial injection abscesses are unusually localised and resolve after surgical drainage with curettage. In other cases, where deeper lesions of the hand and foot have occurred in individuals occupationally exposed to injuries, antimicrobial therapy was required (Grange, 1988).

The introduction of BCG as a vaccine against tuberculosis rarely results in complications (Lotte *et al, 1984*). However, when it occurs the infection ranges from superficial lesions to osteomyelitis and are considered as exogenous in source (Warren, 1984; Peltolta, 1984). For example, the abandonment of the Swedish BCG vaccine in 1979 was a result of an unacceptable incidence of pelvic osteomyelitis in children (Wasz-Hockert, 1979).

Another infection of the skin by mycobacterial species such as M. kansasii seems to resemble M. marinum infection. Although it is well documented as a cause of pulmonary disease, infection of the skin with M. kansasii has been described as resembling cellulitus in a renal transplant patient (Frase, 1975).

A very rare Mycobacterial skin infection is caused by Mycobacterium haemophilum. So far, only a handful of cases have occurred around the world. The first case was reported from Israel (Sompolinsky *et al*, 1978), another five cases were reported from Australia (Mezo A *et al*, 1979) and one case from Ohio, United States (Bryan, R *et al*, 1982). In all cases, the lesions occurred on the limbs which later ulcerated and had a yellow discharge. The ulcer takes a relatively long time to heal and results in the appearances at other sites, which is probably due to direct inoculation from the primary site into other traumatised parts of the skin. All of the reported cases were in immunosuppressed patients following renal transplant, except one case where the infection occurred in a healthy child (Dawson D J *et al*, 1982) and one

patient who was undertaking steroid therapy. Although the source of the infection was not identified, it is believed to have come from contamination of water supplies.

EPIDEMIOLOGY OF M. ULCERANS

Following the discovery of the first case of M. ulcerans infection by McCallum *et al* in 1948, results of experimentation were reported with this new mycobacterium in laboratory animals, and cultural characteristics were established (Tolhurst and Buckle, 1948; Sissons, 1948). Cases were sought out and diagnosed in different parts of the world. The main outbreaks of the infection were reported from Zaire, where over 80 cases of the disease with a similar clinical picture, bacteriology and pathology were seen (Meyers *et al*, 1974; Van Oye *et al*, 1950), in Uganda (Clancey *et al*, 1964), Nigeria (Gray *et al*, 1967), Ghana (Bayley, 1971), Cameroon (Ravisse, 1977), Malaysia (Pettit *et al*, 1966), Mexico (Aquilar *et al*, 1953), New Guinea (Reid, 1967), French Guiana (Grosshans *et al*, 1979) and in at least three different areas in Australia (Radford, 1975). The feature of the reports since the disease was first recognised present a clustering of cases, although sporadic cases do occur. This phenomenon has led to a number of nomenclatures being given, starting with "Bairnsdale ulcer" in Australia, "Buruli ulcer" in Uganda, "Tora", "Kasongo" and "Kakerifu" in Zaire, "Kumusi ulcer" in Papua and "Sik bilong wara sepik" in New Guinea. In one endemic area of Uganda, the local inhabitants called it "the sore that heals in vain" (Lunn *et al*, 1965).

The most obvious, but not invariable, feature of the clustering in this disease is that it tends to be around rivers, swamps and lakes (Radford, 1974; Barker, 1972; Lunn *et al*, 1965). Proximity to, rather than direct contact with, rivers has sometimes been noted (Uganda, Buruli group 1971; Barker 1971). However, occasional reports have been made from forested and inland areas, not obviously associated with large water masses (Glynn, 1972; Pettit *et al*, 1966; Lane, 1964). Another feature of this disease is that it does not parallel population distribution along the water courses. Some outbreaks occurred only after unusual flooding (Barker, 1973; Radford, 1974A).

In reports from Zaire,the disease was most common amongst labourers in mining camps (Janssens *et al*, 1959). The highest incidence recorded was amongst refugees in a camp in Uganda to whom the disease was unknown in their home area and in whom the incidence rapidly disappeared when they moved away from the area (Uganda, Buruli Group, 1969, 1971). It should be noted that the number of cases diagnosed with M. ulcerans infections has risen as clinical knowledge of these lesions has improved, but it is difficult to assess the global incidence of Buruli ulcers given that their detection and study depends largely upon the presence or absence of medical infrastructure in some of these under equipped areas.

Thus Buruli ulcer occurs primarily in the tropical areas and has well defined endemic zones, the largest of which are in Central Africa.

SOURCES AND ROUTES OF INFECTION BY M. ULCERANS

The source of infection with M. ulcerans is still open to question. Many hypotheses have been proposed and numerous attempts to isolate organisms from non human sources have failed to yield pathogenic M. ulcerans. One hypothesis which finds strong support is that the organism has an environmental niche from which it opportunistically infects humans. Isolation of large numbers of fast and slow growing mycobacteria have been made from grasses and sedges in areas of Uganda and Zaire, but without M. ulcerans being recognised. These organisms were more common on vegetation and in permanent swamps. This is where most cases of M. ulcerans infection occur. On the basis of these findings, human infection is postulated from contact with this vegetation, and subsequent penetration of the organism is thought to occur through small scratches or other traumatic lesions in the skin (D.J.R Barker 1972, Stanford 1973). The argument that the skin can be injured by many things in addition to plants, leading to subcutaneous tissue infection, was made by Meyers (1974). He considered the possibilities of other reservoirs such as the commensal flora of cold-blooded animals such as reptiles and frogs which can come into contact with man directly or constitute a source of environmental contamination. Fish, frogs, toads and mammals have been proposed as possible reservoirs by Radford (1975) in New Guinea. Rodents captured in Uganda presented numbers of mycobacterial infections, but M. ulcerans has never been isolated from them (Revill, Morrow, Parson and Kiryabawire, 1972). Some arthropods, such as mosquitos, flies and scorpions have

also been proposed as candidate vectors (Radford 1975). Bed bugs found on people and bats which are particularly common in parts of Australia have been looked at as a possible vector (Buckle 1972). Despite all the efforts put in to the search for M. ulcerans and its possible reservoirs, the organism has not yet been found.

On the basis of the current knowledge of leprosy transmission, in a review of cases of M. ulcerans infection in Zaire, Pattyn (1973) hypothesised that transmission might be air borne followed by haematogenous dissemination. This is not proven and the main route of transmission is still believed to be direct inoculation of the organism into the skin.

A strong link between water and the increased number of cases has been found in Uganda. Reported cases mainly come from the Nile Valley and bordering marshes (Lunn *et al*, 1956; Connor and Lunn 1966; Barker 1972a,b). The swampy areas of French Guiana are reported to have cases of M. ulcerans infection (Pradinoud, 1974). In Zaire the disease was found to be confined to the Savannah areas (Portaels, 1970). Also cases were found near a water source (Meyers 1974). In New Guinea, most cases came from villages along the Kumusi stream especially after major floods (Radford 1974). However, a weaker link between water and reported cases is observed in Australia (Radford, 1975).

CLINICAL DISEASE CAUSED BY M. ULCERANS

The clinical features of the infection are distinguishable from any other mycobacterial disease. Mostly ulcers occur on the extremities and there is frequently a history of minor injuries which fail to heal (J. Hayman, 1985).

A typical infection develops in the subcutaneous tissue and is followed by necrosis. An ulcer characterised by undermining of the margins and a sharply defined ulcer edge develops (J. Hayman, 1985). Beyond this area of thickening, the skin appears normal.

The initial lesion of light coloured skin causes reddening, in darker skins some increase in colour may occur (Radford 1974). Hypo-pigmentation follows with thinning of the epidermis which may result in multiple breaches in the skin surface (Radford 1974). The peripheral edges of lesions are commonly hyper-pigmented. Large necrotic areas sometimes develop with subsequent separation of frankly gangrenous tissue. Satellite lesions may occur, from which tracks to the initial area can usually be defined by probing (Radford 1974).

At the outset, lesions may itch but ulcers are characteristically painless unless secondary infection is superimposed, the lesion involves deeper structures, or it metastasises. Oedematous lesions are often painful (Radford 1974B; M Grange 1988; J Hayman 1985). Extension of the ulcer takes place, often asymmetrically, over weeks or months,

and tends to occur more rapidly in children. The edges are undermined. This undermining may be very extensive and can exceed 10 centimetres in any direction (Radford 1974B).

Another characteristic of lesions is their tendency to remain superficial to the deep fascia, but extend below it in up to 20% of cases (Radford 1974B). Connective tissue, muscle, bone and nerve have all been known to be directly involved. Lesions without secondary infection are cold (Radford 1974B). Multiple lesions occur in 1-2% of cases in Uganda (Revill 1973a; Barker 1972) and in 4-6% of patients in Papua New Guinea and Australia (Radford 1974B, 1975). A high prevalence of multiple lesions (up to 20% of cases) has been noted in Zaire. The metastatic bone lesions present with pain and can occur after the initial lesion has healed (Janssens *et al* 1959; Lunn 1963).

In lesions where extensive involvements of fatty tissue is visible, tissue takes on a whitened appearance. Calcium is sometimes deposited in the fat giving it a gritty sensation to touch and a sensation on incision like that of cutting a scirrhous carcinoma (Radford 1974B). Acid-fast bacilli are more commonly found in smears from necrotic areas or undermined edges. Re-epithelialisation of edges is sometimes seen in lesions of long standing, but is occasionally seen quite early. Healing may occur in one area while extension develops in another, occasionally ulcers undergo complete spontaneous regression. If this happens after extensive ulceration has occurred, scarring contractures and even ankylosis may result (Radford 1974B). Systemic upsets with

fever and toxaemia are rare and probably occur only as a result of secondary infection with pyogenic organisms. Similarly, lymphadenitis has not been reported in humans except in association with secondary infection. M. ulcerans organisms have never been recovered from lymph glands (Radford 1974B).

The natural history of this disease is for a change in immune response to occur accompanied by healing of ulcers, disappearance of bacilli, resolution of oedema and extensive fibrosis. A late result of this fibrosis is contraction, which may severely limit the future use of a limb. Thus the disease is one of low mortality, but of high morbidity if it is not treated.

A J Radford (1974B) described three distinct types of clinical lesions:

a) Papulo-necrotic lesion - the commonest form;

b) Oedematous lesion - less common. The skin in such lesions becomes tight and passes through a spectrum of colour change from violet to red to brown to necrosis and finally moist gangrene about a month later (Janssens *et al*, 1959);

c) Metastatic lesions - rare form. Usually involve metastatic spread to ipsilateral and contalateral sites, usually proximal to elbow and knee joints. In Uganda it is uncommon, but in Zaire (Janssens *et al* 1959; Pattyn 1973), it occurs in 20% of cases and may involve peripheral bones and joints.

On the basis of histological examination of very early lesions, the Uganda-Buruli group describe two types of lesions:

a) **Reactive Type**

In eleven out of thirty-two cases epithelial and giant cell systems of the tuberculoid type with very few acid-fast bacilli were reported. A strong host response is observed by positive reaction to skin testing.

b) **Non Reactive Type**

This is a more frequent, non-reactive lesion showing extensive subcutaneous necrosis of fat with or without ulceration. No granuloma formation and minimal cellular filtration. Large numbers of extracellular acid fast bacilli were present.

IMMUNOPATHOLOGICAL SPECTRUM OF M. ULCERANS INFECTION

Like leprosy, M. ulcerans disease has an immunological spectrum, although this differs from that of leprosy in that each patient passes through the whole spectrum. In the ulcerative and pre-ulcerative phases of M. ulcerans infection there are large numbers of bacilli, little histological reaction, negative lymphocyte transformation and negative skin test, very similar to the situation in lepromatous leprosy.

In the healing phases of M. ulcerans infection there is a massive non-casceating granulomatous response around few or no bacilli, in some respects similar to tuberculoid leprosy.

A very interesting aspect of M. ulcerans infection is this spontaneous change from a lepromatous appearance to a tuberculoid one, showing that the pattern of natural recovery from disease is immunologically mediated.

Like both tuberculosis and leprosy, many persons become skin test positive to Burulin without ever developing disease. These observations show that immunisation against M. ulcerans infection may be possible.

The sequence of events occurring during the normal course of infection is described by Dodge (1964) and Connor and Lunn (1966). Before the stage of ulceration, acid-fast bacilli are found in the centre of a necrotic mass of fat, diminishing towards the periphery and absent in the viable tissue. Later, following ulceration, the bacilli are found predominantly in the deep zone of the necrotic mass. When the process of phagocytosis takes place, a discrete granuloma of foamy macrophages occurs and later there is evolution of epithelioid cells and giant cells. The healing stage is characterised by granulation tissue filling the whole of the defective areas which later becomes converted by epithelial cells overlaying fibrous tissue. At this stage, lymphocytes are numerous in the lesion (Ridley *et al*, 1978). The nature of this histological spectrum is dominated by massive tissue necrosis associated with excretion of soluble toxin, which may spoil the development of the classical tuberculoid granuloma (Read *et al*, 1974). Connor and Lunn (1966) distinguish four types of granuloma:

a) Diffuse macrophages which represent the phagocytosis of necrotic fatty tissue.

b) Foreign body granuloma in the superficial dermis, which they attribute to the presence of foreign matter which has entered the open ulcer.

c) A reaction to the keratinous contents of epidermal cysts, due to trapping at the periphery of the ulcer.

d) The tubercle-like granuloma which develop around the resolving necrotic mass. This type of granuloma is not concentrically organised, the epithelioid cells are loosely arranged and appear to be immature.

TREATMENT OF M. ULCERANS INFECTION

The main approach to therapy in M. ulcerans infection is based on excision of the ulcer. This should include an area of macroscopically normal skin, followed by split skin grafting to the denuded area. Because many cases of M. ulcerans infection occur in isolated areas where surgical, anaesthetic and transfusion facilities are minimal, the approach of extensive curettage rather than radical excision has been recommended (Shattock, 1965).

Lunn and Rees (1964) found they could reduce the size of surgical procedure and decrease the hospitalisation time by three months using curettage combined with the administration of clofazimine with or without streptomycin.

Radford, 1974B, found most lesions can be adequately treated under regional perfusion anaesthesia with the use of tourniquets. The only excision undertaken was of tissue which was not viable. Split-skin grafts were applied to those areas where primary closure could not be effected.

Locally applied treatments have been tried. They include PAS solution and powder, Etisul, Eusol and 0.25-0.5% silver nitrate solution. Results were variable (Forbes, Wannon, Kirkland 1954; Gray, Kingmaand, Kok, 1967). As a rational adjunct to surgery, the application of local heat to raise the temperature of the infected area to 40°C in order to discourage growth of organisms has been recommended (Melmeney and Johnson 1950; Francis 1957; Lunn 1963; Reid 1967; Glynn 1972).

Orally administered anti-mycobacterial agents have been used with some apparent success, at least in halting spread, if not curing, the lesions. Pettit, Marchette and Rees (1966) reported total healing of the lesions with clofazimine when it was used continuously for a period of up to six months, but produced no evidence that healing was due to the drug.

A trial of clofazimine compared with an inert placebo in over 100 patients showed no statistically significant difference between the two substances, nor was there any evidence to suggest any benefit from clofazimine either alone or with surgery (Revill, 1973b; Revill *et al*, 1972). A trial carried out by the same author suggests a slight, but not statistically significant, benefit of rifampicin when compared with clofazimine.

Using experimental M. ulcerans infection in mouse foot-pads, rifampicin showed a temporary delay in the progress of early and advanced lesions. Clofazamine alone (15mg/kg/daily) or with sulphadoxine in drinking water failed to produce any improvement in the course of the infection. (Stanford and Phillips, 1971).

Relapses after what appear to be cure are uncommon but do occur. Revill, 1973B, reported a series of 272 patients in Uganda with an 8% recurrence rate at the same site after apparent healing.

It is interesting to note from the studies described above that there may be a relationship between the different stages of the disease and the success of treatments applied. In the early stages of the infection and for secondary infection during the healing process, anti-bacterial agents are probably useful. In the stage when the bacilli are large in number and surrounded by masses of necrotised fat, anti-bacterial chemotherapy is less effective. Surgical removal of the dead tissue and locally applied treatment may be most useful at this stage. Corrective measures, such as local heat and skin grafting, are probably most useful in the long drawn out healing phase.

DRUG SENSITIVITY IN VITRO

Glynn, 1972, found little reason to test isolates for drug sensitivity because the disease is refractory to chemotherapy. Nevertheless, clinicians usually request testing of isolates. Strains isolated in Papua New Guinea and tested by David, 1974, using the resistance ratio method, were found to be sensitive to streptomycin, cycloserine, capreomycin and rifampicin, but there was no mention of the concentrations used.

In another larger study by Clancey, 1964, sixteen strains from Uganda were tested for sensitivity to:

1. Streptomycin: M. ulcerans were completely inhibited only at a concentration of 64ug/ml whereas M. tuberculosis strain H37RV inhibited at 4ug/ml.

2. P-Aminosalicylic acid: M. ulcerans developed a pattern of resistance between 0.5ug/ml to 4ug/ml but were completely inhibited at 8ug/ml. M. tuberculosis H37RV were inhibited at 0.5ug/ml.

3. Isoniazide: Five strains out of sixteen were sensitive to a concentration up to 50ug/ml whereas M. tuberculosis H37RV were sensitive to 0.1ug/ml.

4. Viomycin: All M. ulcerans were completely resistant to a range of concentration (5ug-80ug/ml). M. tuberculosis H37RV were inhibited at 80ug/ml.

5. Ethionamide: Ten strains were inhibited by 80ug/ml and another six were inhibited by 160ug/ml. M. tuberculosis H37RV were inhibited by 80ug/ml.

Calculating the resistance ratio of the above suggests that ethionamide has the lowest resistance ratio - R.R. = (1)

$$RR = \text{Minimum inhibitory concentration of the } \frac{\text{test strain}}{\text{control strain}}$$

to those strains inhibited by 80ug/ml: the R.R. = (2)

to those strains inhibited by 160ug/ml): the R.R. = (4)

For the other anti-mycobacterial agents, M. ulcerans is considered highly resistant. Streptomycin and P-aminosalicylic acid have resistance ratios of 16 and isoniazid has a resistance ratio of 500.

Despite the similarity of mechanisms of action of ethionamide and isoniazid, both are inhibitors of mycolic acid synthesis (Winder and Rooney, 1970a; Winder *et al*, 1971). Ethionamide appears to be far more effective.

A study on other mycobacterial species carried out by Rist, 1960, as the two drugs appear to act on the same site in the mycolic acid pathways. Ethionamide action is direct, whereas isoniazide acts only after being processed by catalase-peroxidase, ie. strains of mycobacteria which lack catalase-peroxidase activities are resistance to isoniazide and sensitive to ethionamide. This corresponds with the negative biochemical tests for catalase and peroxidase in M. ulcerans.

Looking to other anti-mycobacterial agents for their action against mycobacteria, such as:

a) Rifampicin and streptomycin are inhibitors to DNA-dependant-RNA-polymerase and have a more delayed effect on protein synthesis (Goldberg and Freidman, 1971).

b) Streptomycin is an inhibitor of protein synthesis, occurring on 30S sub units. This was shown by resistance studies on mycobacteria (Tanuka 1975; Schlessinger and Medoff 1975).

c) Neomycin and kanamycin have similar mechanism of up-take to streptomycin and inhibition of protein translation at the 30S sub-unit (Tanaka, 1975).

d) Viomycin and capreomycin are other protein inhibitors of mycobacteria which block polypeptide synthesis and translation of t-RNA (Choi *et al*, 1979).

All of these antibiotics require binding and penetration of the cell wall before they can reach their intended target. Studies by Notle showed that resistance to streptomycin, kanamycin and viomycin in mycobacteria was due to the inability of the resistant strains to bind the antibiotic to the cell wall and transport them across a permeability barrier (Notle, 1964; Bonicke, 1970). It was also found in studies by Beggs and Williams, 1971, that the initial binding of radioactive streptomycin to the cell wall is a passive process inhibited by enzyme poisons. Studies by Lederer, 1971; Lederer *et al*, 1975, found that ethionamide acts on the mycolic acids which are part of the cell wall structure. Since M. ulcerans is regarded as a biochemically inactive and very slow growing organism, whether *in vitro* or *in vivo*, the process of cell division, protein, DNA and RNA synthesis may take longer than is expected of other mycobacteria.

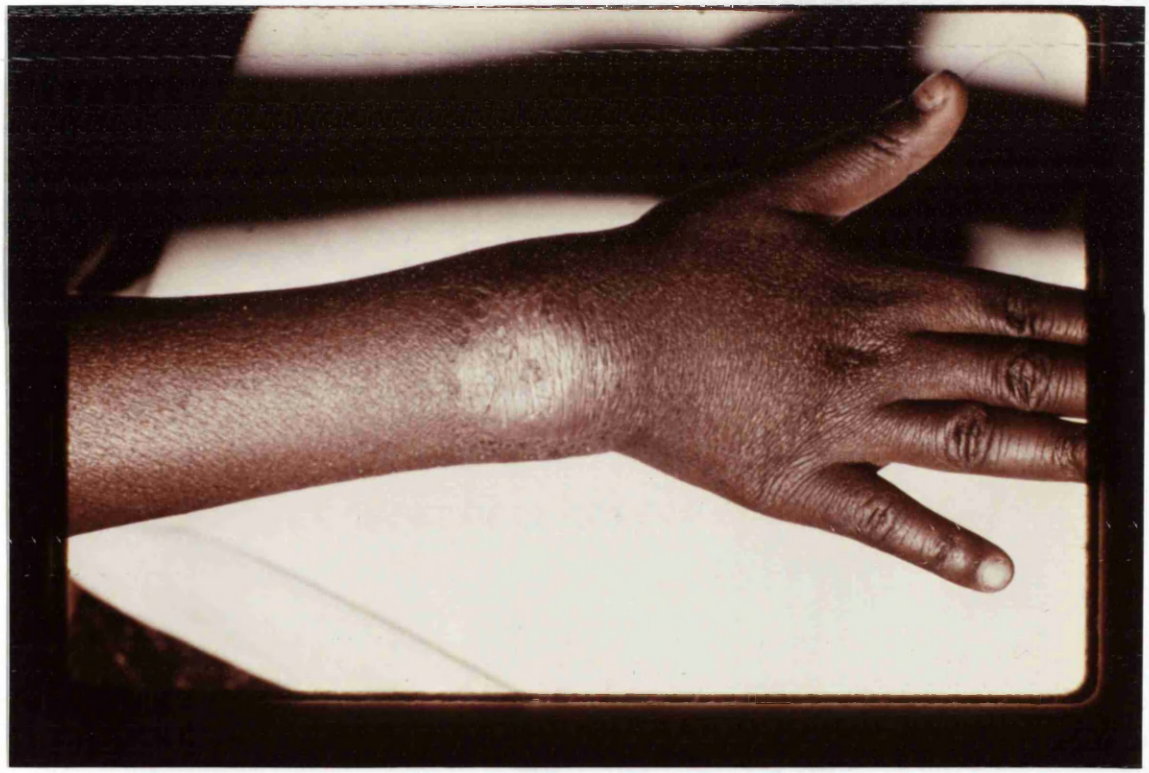
Therefore, antibiotics fail to get to their target and, if they do, they are prevented by the dead tissue surrounding the bacilli from doing their action. In a study by Nalin *et al*, 1981, it was found that one division cycle of M. avium requires ten hours to be completed on a rich medium. The high resistance of M. ulcerans to anti-mycobacterial drugs *in vivo* has not yet been subjected to any scientific study, therefore a clear guideline on therapy does not really exist.

PREVENTION

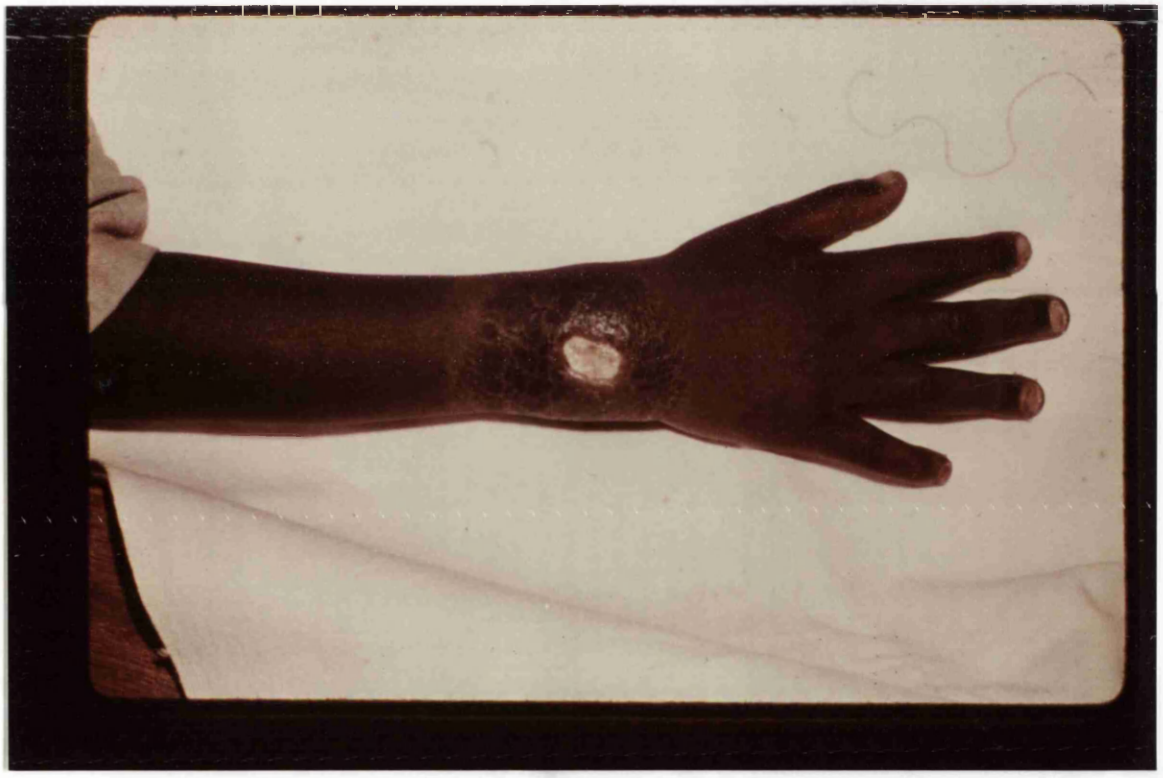
Without knowledge of either the natural reservoir of infection or the route of transmission, preventive measures are difficult to introduce. Uganda (Buruli group 1969) based its prevention programme on education and early recognition of cases.

In two controlled trials in an endemic area of Uganda, BCG was used as a protective measure against M. ulcerans infection. The first trial was carried out by Uganda Buruli group, 1969, but this trial was brought to a premature end when the settlement left the endemic area. Nevertheless, the results obtained during this two year trial (1968-69) suggested that BCG produced a 50% over-all, short-lived protection. However, long-lasting protection and delay in onset of the disease was observed. The vaccination trial carried out by Smith *et al*, (1976) which lasted for a longer time (1970-1974), concluded that BCG vaccination offered 47% protection which later started to decline to little or nothing after a period of six to twelve months.

Even though BCG produced considerable protection in Uganda against leprosy and tuberculosis, these two trials clearly suggest that BCG has no significant protective effect against M.ulcerans disease. The longest controlled study was carried out in Papua New Guinea; it started in 1962 and was followed until 1971. The results of this trial showed a decline in cases which was explained by the immunisation campaign having coincided with a fall in the secular trend of the disease (Radford, 1974). Thus, evidence from the literature suggests that vaccination and anti-mycobacterial therapy cannot clearly establish the role of protection or cure against M. ulcerans disease, and the problems remain unresolved.



Photograph 1: Early stage of M. ulcerans infection in Man



Photograph 2: Late stage of M. ulcerans infection in Man

CHAPTER FOUR

IDENTIFICATION OF M. ULCERANS

INTRODUCTION

The schemes of mycobacterial identification were developed through collaborative studies undertaken by the International Working Group on mycobacterial Taxonomy (Wayne *et al*, 1971; Kubica *et al*, 1973; Meissner *et al*, 1974; Saito *et al*, 1977). As a result of these studies, several philosophical approaches were adopted, such as the scheme of Marks (1976), which is based on identification of the strains as far as is necessary for the proper management of the patient. The strains were allocated to groups rather than species, and if further identification is required for research or epidemiological purposes, then more refined techniques can be used. Under this scheme, laboratory identification of M. ulcerans is not clear and sometimes is misleading, as they consider M. ulcerans in their psychrophiles group.

TABLE 1

IDENTIFICATION OF M. ULCERANS ACCORDING TO MARKS (1976)

Ulcerans Group	Temperature Range °C			Oxygen Preference	Pigmentation Dark/Light	
	25	37	45			
M. ulcerans	+	±	-	micro	-	-
Unclassified	+	±	-	micro	-	-

The principles used by Pattyn and Portaels (1972) provide the most accurate and clear system for M. ulcerans identification. The strains are first identified as rapid or slow growers with consideration of the morphology and pigmentation.

TABLE 2

IDENTIFICATION OF M. ULCERANS ACCORDING TO PATTYN AND PORTAELS (1972)
(Based mainly on Zairean strains)

Pigment Production	Photochromic	-
	Scotochromic	F
	Nonchromic	M
Temperature Range(°C)	33	M
	37	F
	42	-
Resistance	Isoniazid (10ug/ml)	M
	Thiophene 2-Carboxylic acid hydrazide (1ug/ml)	+
	Hydroxylamine hydrochloride (250ug/ml)	F
	p-nitrobenzoic acid (500ug/ml)	-
Biochemical Tests	Catalase 45mm	-
	Niacin production	-
	Nitrate reduction	-
	Acid phosphate	-
	Tween 80 hydrolysis	-
	Beta-galactosidase	-
	Growth in 5% (w/u) NaCl	-
	Urease	F
	Nicotinamidase	F
	Pyrazinamidase	F
	Colonial morphology	R

Keyboards:

+ = > 85% are positive
- = < 15% are negative
M = 50% to 85% of the strains are positive
F = 15% to 49% of the strains are positive
R = Rough appearances

The basic type of colony was well described and illustrated for several species by Fregnan and Smith (1962) and by Vestal and Kubica (1966). They are rough and smooth, which seems to depend on the presence or absence of capsular materials produced by the cell wall. Most freshly isolated strains of mycobacteria commonly grow as smooth colonies (Tsukamura, 1976) and rough mutants arise during subcultivation (Fregnan *et al*, 1962; Stanford and Grange, 1974). Different forms of colony (artificially produced mutants) can be produced, such as the reported study by Kolman and Ehrenderg (1978) where colonies of M. phlei resembling shuttlecocks have been produced.

Thirty two strains of M. ulcerans were obtained for my study, 10 came from my superviser Dr Stanford, the remaining strains were kindly provided by Professor F Portaels and by Dr Hugo David. These are shown in Table 3.

All thirty two strains of M. ulcerans used in this study were examined by different methods to confirm their identity.

TABLE 3

COLLECTION OF MYCOBACTERIUM ULCERANS STRAINS USED IN THIS STUDY

Strain No.	Isolated by	Source	Mouse Pathogenecity	Growth on Sauton	Other Ref.
109 0002	Dr Lavalle	Mexico	+	-	
109 0004	Dr Barasse		+	-	
109 0007	Dr Pattyn	Zaire	+	-	
412	Dr Stanford	Zaire	+	-	2097/Kiuslora
413	Dr Stanford	Zaire	+	-	2098/Kiuslora
421	Dr Stanford	Zaire	+	-	2106/Kiuslora
109 0009	Dr Huet	Cameroon	-	+	
109 0011	Dr Huet	Cameroon	+	+	
109 0012	Dr Huet	Cameroon	-	-	
109 0013	Dr Huet	Cameroon	+	+	
7748	Boisvert	Cameroon	+	+	
7749	Boisvert	Cameroon	+	+	
7750	Boisvert	Cameroon	+	+	
109 0015	Dr Grosset	France	+	+	
851	Dr Stanford	Congo	+	-	
298	Dr Stanford	Uganda	+	-	TC 181
340	ITM		+	-	
4253	Moscateli		+	-	
5245	Kimpese	Zaire	+	-	
5566	ITM		+		
7730	Dawson	Australia	+	+	
7832	Dawson	Australia	+	-	
7833	Dawson	Australia	-	+	
21789	K. Jackson	Queensland	+	-	
8471	K. Jackson	Queensland	+	-	
17680	K. Jackson	Victoria	+	-	
842	van Keulan		+	-	
1459	Clancey	Uganda	+	+	NCTC 10445
418	Dr Stanford		+	-	R S Fever
7634	-	-	+	+	
5016	-	-	+	-	
101	-	S. America			

A. ISOLATION OF THE ORGANISM FROM CLINICAL SAMPLES

Diagnosis of M. ulcerans cases is largely based on the clinical picture and treatment does not depend on the results of laboratory identification since chemotherapy is regarded as non-effective. Isolation of the organism is mainly useful for research purposes. The most important stage in the process of isolation of M. ulcerans is the collection of the specimen. In lesions caused by other mycobacteria, the organism can usually be cultured and stained for acid fastness by taking a swab or a biopsy from the centre of the lesion, but in some cases where the clinical features of M. ulcerans infection were found, the organism had never been isolated. This can be explained as the centre of the lesion often does not contain enough organisms to culture or to demonstrate by staining. The undermined edges of the ulcer is where the organism can be found in large numbers. The other problem which occasionally contributes to the failure of isolation is contamination with other environmental mycobacteria. Since M. ulcerans is a very slow grower, especially during primary isolation, other mycobacteria such as M. gordonae and M. scrofulaceum can overgrow and hide the real cause of the lesion (Mitchel *et al*, 1984; J Hayman 1984). I have experienced this problem during the isolation of the organism from ulcerated mouse foot-pads.

B. BACTERIOLOGY

Cultural Characteristic

Colonies of M. ulcerans on Lowenstein-Jensen (LJ) medium (Figure 1) appear as small pale domes, later becoming lemon or creamy-yellow in colour. In some strains, continuous subculture on LJ medium leads to a smooth appearance, with a degree of loss of pigmentation. However, when the organism is recultured from infected material, it regains its rough morphological appearance with deep yellow pigmentation.

Growth Conditions

Not all strains were able to grow on Sauton's medium, but those which did grow and were continuously subcultured preserved the rough appearance and deep yellow pigmentation. Isolation of the organism from infected material (mouse foot-pad) will only take place on LJ medium. It seems an essential ingredient is present in LJ medium which is required for primary isolation, because even those strains which were able to grow on Sauton's after subculture from LJ, did not grow on Sauton's medium directly from infected material. Also I found, Oxoid blood agar base with 7% difibrinated horse blood is another useful media for primary isolation. This medium is mainly used for cultivation of fastidious organism such as Clostridium and Haemophilus species.

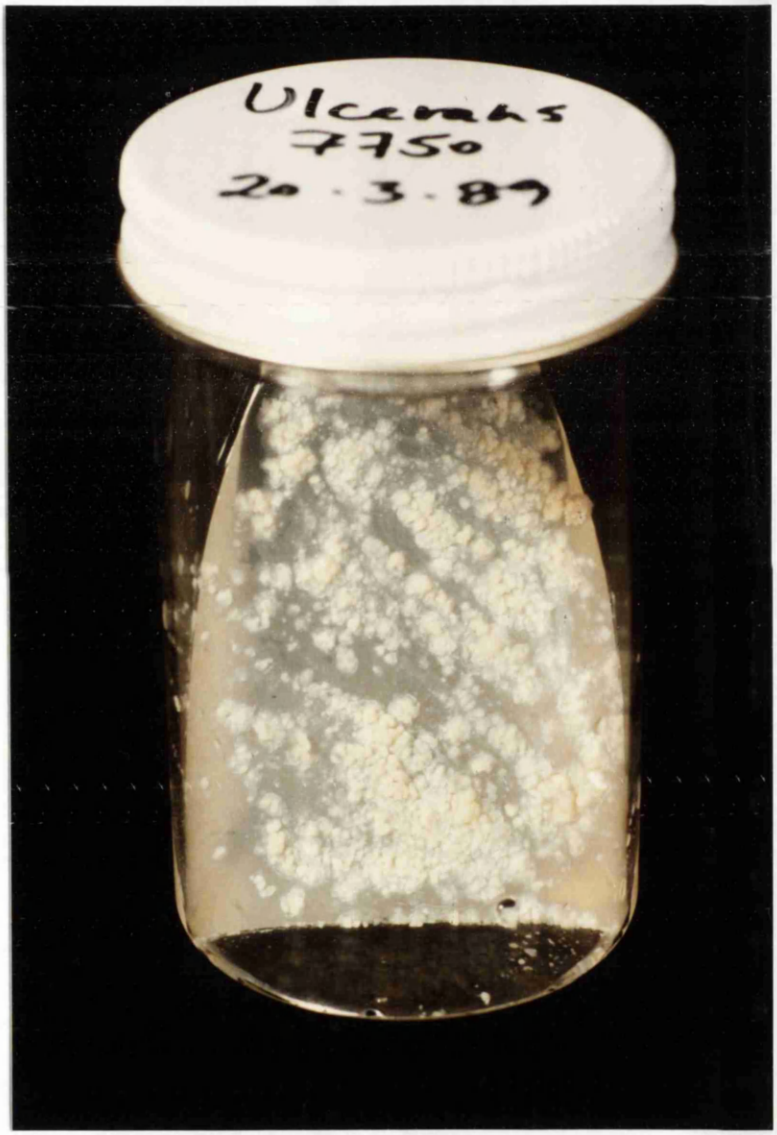


Photo (1): M. ulcerans strains No. 7750 grown on Sauton's media Approximately 10 weeks after incubation at 32°C

The strains which developed good growth on Sauton's medium were used to prepare Burulin (Stanford, *et al* 1975), a specific skin testing reagent for M. ulcerans. This was used to follow the different stages of the disease, (which will be described later) by foot-pad sensitivity testing.

The time required to see the colonies on primary isolation depends on the quantity of the organism in the cultured sample. However, 3-4 weeks is the minimum time before any visible growth can be seen.

A very significant characteristic of M. ulcerans is the temperature range over which the organism is able to grow. This is an important diagnostic tool. All of my strains grew at 32°C, although some strains were able to produce just visible colonies after about six months on LJ medium at room temperature. None of the strains grew at 37°C or 25°C. However, in some studies a few successful cultures at 37°C have been made in Zaire, where the disease showed some unusual features (Pattyn; Pattit *et al*, 1965). Most of these strains were isolated from bone lesions with heavy infiltration by the organism.

Microscopic Examination

Suspensions of the organisms were made in PBS buffer. It is often difficult to obtain a smooth suspension from an old culture. Films were made on 3 x 1 inch glass slides and allowed to dry. The slides were stained by the traditional Ziehl-Neelsen method. Carbol fuchsin stain is poured over the slides so that the smear is completely covered

and the slides are then gently heated from below. The stain is allowed to act for 2-3 minutes. They are then washed in running water and decolourised by 15% H_2SO_4. Malachite green was used as counterstain, poured on the slides and left 1-2 minutes to act, then washed off. The slides were allowed to dry and examined under oil immersion. The bacilli are very strongly acid-fast and morphologically are indistinguishable from M. tuberculosis, although they do not form coils. In direct smears from mouse foot-pad material the organism appears in clumps, single bacilli or pairs. Beading of the bacilli at one or both ends has been noted. Direct smears of culture under the microscope appear as irregular clumps with very few isolated bacilli visible.

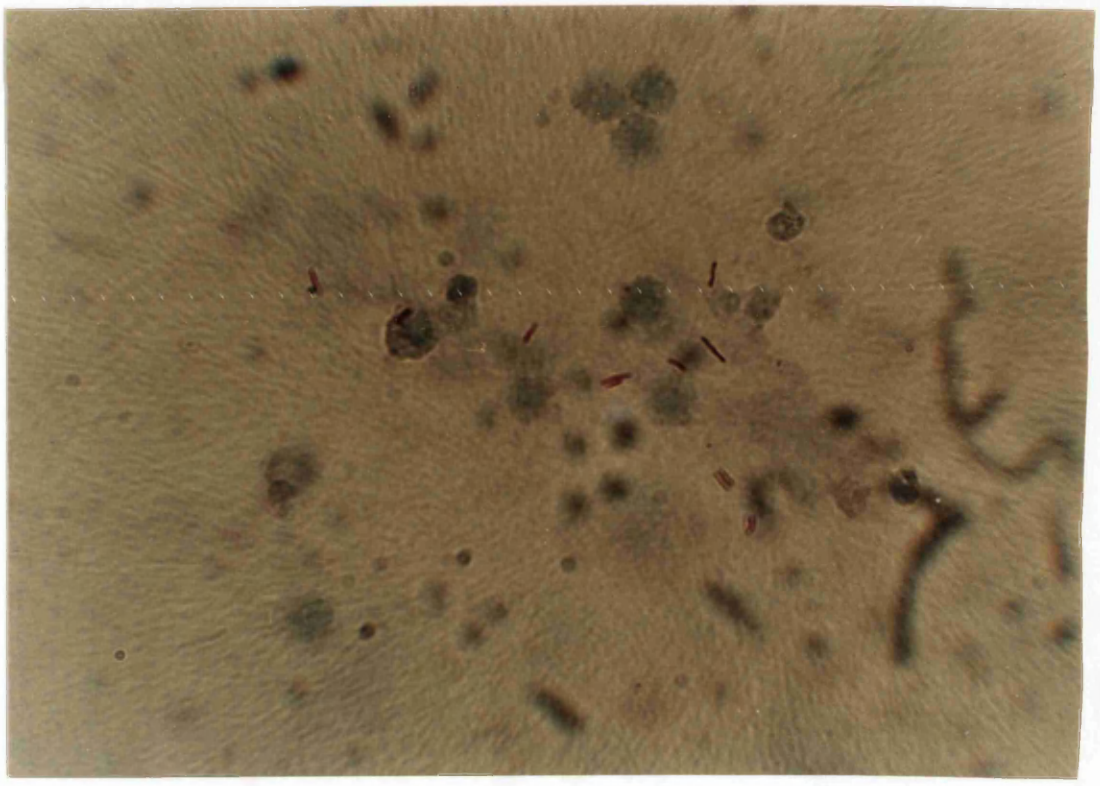


Photo (2): Ziehl-Neelsen staining of M. ulcerans demonstrated under light microscopy using oil immersion lens.

C. INFECTION IN ANIMALS

The other most important feature in the bacteriological identification of M. ulcerans is mouse foot-pad inoculation. This is useful in confirming the identify of the strains and used for research purposes (Pattyn, 1965).

MATERIAL AND METHODS

Infection in Mice

The mice used in the experiments carried out in this project were Balb/C females aged between six to ten weeks. C57 mice which are a very sensitive mouse strain to M. ulcerans infection were used when no response was observed in Balb/c mice. This was done in the case of three strains (00012, 0009, 7833). A single cell suspension of each strain was made in PBS buffer, and 0.1ml containing approximately 105 organisms, injected into foot-pad.

Preparation of Single Bacterial Suspension

A suspension of single cells was prepared by passage through a small aperture (1ml syringe pressed to the bottom of a 20ml universal). The crude suspension was then transferred to another universal, centrifuged at 10 x 1000 rpm for 3 minutes. The supernatant was taken, counted using an haemocytometer and used for the foot pad injections.

Extraction of the Organism from Foot-pad

A positive response is recognised by swelling occurring 3-4 weeks after injection of the organism depending on the virulence of the strain. To avoid contamination with other mycobacteria, the organism was extracted before the stage of ulceration.

After scraping the foot-pad tissue, the material was ground in PBS buffer in Griffith's tubes containing sterile sand. The supernatant was collected in a universal tube and 2ml of 10% oxalic acid added for decontamination (Corper H and Uyel N, 1930). The mixture was allowed to stand for approximately 15 minutes with occasional shaking before centrifuging for 5 minutes at 10 x 100 rpm. The deposit was cultured on LJ medium and incubated at 32°C and 37°C. A smear of the deposit stained with Ziehl-Neelsen showed a large number of acid-fast bacilli.

A comparison was made between M. kansasii from mouse lung, and M. tuberculosis (Bovine type) from vole lung, contrasting with the scanty Bacilli in these preparations the enormous number of M. ulcerans in the mouse foot-pad is very obvious (photos 3,4 and 5). Note too, the difference morphologies of the three species. M. kansasii appears like "banded snakes" and the bovine organism are short, darkly stained and with granules of uncertain nature. The M. ulcerans are more evenly but less densely stained and this tends to be typical for them. It would have been interesting to mix the three organisms in the same smear to check that these characteristics are retained even when the staining procedure is precisely the same. The photographs are not comparable in respect to size of organism.

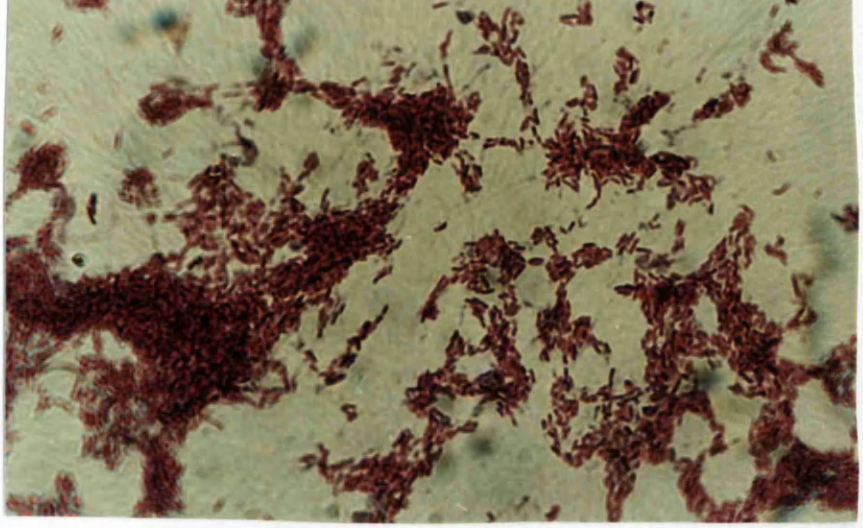


Photo (3): M. ulcerans preparation from mouse foot-pad stained with Ziehl-Neesen

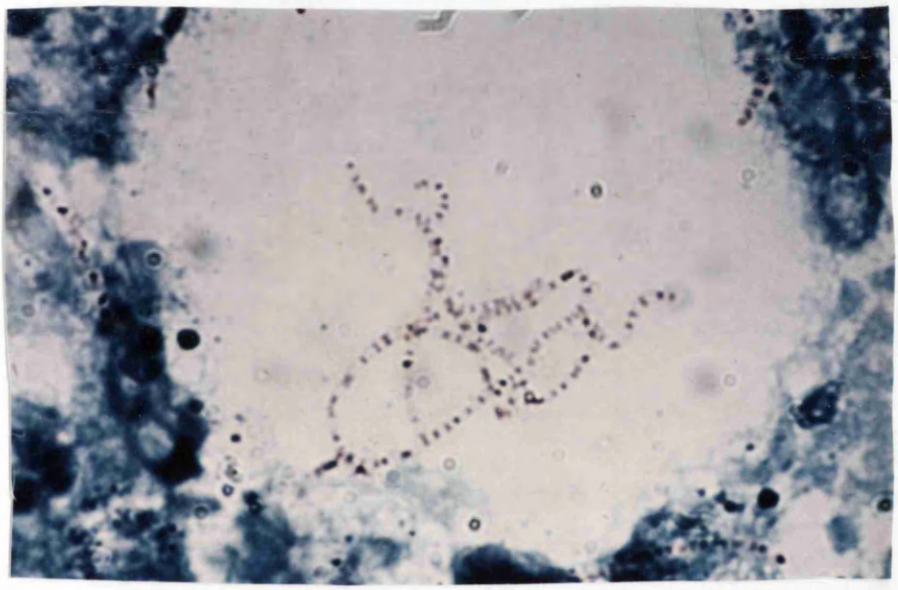


Photo (4): M. kansasii preparation from mouse lung

Photo (5): M. tuberculosis preparation from vole lung

Preparation of Burulin

This skin testing reagent was prepared from M. ulcerans and described in 1975 by Stanford *et al*. The method used to prepare Burulin for this project differed from the method originally used by Stanford. After successfully adapting six strains to grow on Sauton's medium, the process of avoiding egg particles carried over with the organisms from LJ medium was no longer required.

Description of the Method

Strains No. 7748, 5566 and 0009 were used for the preparation as they produce good growth on Sauton's medium. The organism were harvested by scraping them from the surface of the medium and suspending them in M/15 phosphate buffer (pH 6.8). The suspension was subject to ultrasonication for 15 minutes, with the amplitude set at 8-10um in an MSE disintegrator. The resultant mixture of residual whole organisms, broken cell walls and cytoplasm was then centrifuged at 10,000 rev/min for one hour. The supernatant was filtered serially through 0.45um and 0.22um sterile membrane filters.

Natural and Experimental Infection in Animals

It is common to find animals in the wild infected by mycobacteria, the most commonly isolated organisms being M. intracellulare and M. avium, but the most important infection that poses serious threats to the agricultural industry and consequently, a major risk to public health,

is tuberculosis of cattle caused by M. bovis. Although the incidence of this disease has dropped dramatically in recent decades, outbreaks do occur when cattle come into contact with infected sources.

The only cases of natural infection by M. ulcerans in animals was found in wild koala bears captured near Bairnsdale, Australia (Mitchell 1984; McOrist 1985). In the later report, M. ulcerans was involved in respiratory tract infection in these animals. This part of Australia is considered an endemic area for M. ulcerans. Contact between humans and koala bears is highly unlikely. The main source of infection for the human, and presumably the koala, remains the environment (Mitchell *et al*, 1984).

Experimental infection of rats and mice only occurs in the extremities, tail and feet (MacCullum *et al*, 1948), and even if the organism is injected intravenously, the disease only appears in these sites. This is attributed to the lower temperature of these sites being 5 to 10°C lower than the interior of the body, which is in accordance with the restricted temperature range required in order to obtain successful growth. Among other experimental animals inoculated with M. ulcerans, ulcers have been produced in Korean chipmunks (Lew, 1976), 9 banded armadillo (Walsh *et al*, 1975). Mastomys natalensis, a multimammate rodent, has been proposed as a new animal model for M. ulcerans research (Singh *et al*, 1984). Also Anole lizards were infected by M. ulcerans using subcutaneous Route (Maralis et al 1975).

Several factors can affect the level and spread of mycobacterial

infection in natural animal communities, and the occurrence of disease in a population may not reflect the true susceptibility of the species. A good example was noted in monkeys. Tuberculosis in wild monkeys is not usually found but its incidence in captive monkeys is very high and the death rate from tuberculosis can be as high as 10% per annum (Francis, 1975).

Clinical Manifestation of M. ulcerans Infection in Mice

As in human disease, the clinical features of the infection in mice have a distinctive sequence of events. A response to challenge is first recognised by swelling which appears 3-4 weeks after inoculation, depending on the inoculum size, virulence of the strain and the type of mice used. Usually Balb/c mice are more resistant to the infection than C57 Bl strains. Local oedema progresses to involve the whole leg and ulceration occurs at this stage, followed by secondary infection. At late stages, the mice die of extreme wasting in most cases, or with terminal whole body oedema, the so-called "fat mouse" syndrome. Modern ethical consideration prevented me from seeing mice in this late stage of disease.

All M. ulcerans strains were inoculated in Balb/c mice and the classical picture of the disease manifestation was seen with all but three strains. The progress of the infection was assessed by measuring the swelling with a spring loaded engineer's micrometer graduated in 1000's of an inch (Table 4). Strain No 0009 which caused transient swelling at 3 weeks and strains No. 00012 and 7833 which did not cause

any response are the exceptions. These three strains were re-inoculated in C57 Bl mice and the same results obtained. (See Photo (6).

A large number of acid fast bacilli were seen when microscopic examination of tissue from foot-pads was carried out 5-6 weeks after the initial inoculation with the exception of the three strains which did not produce the typical disease.

Foot-pad inoculation in rats produces similar results in mice, but guinea pigs and rabbits are not susceptible to the infection (Clancey, 1964). The immunological events of the disease in man and mice will be discussed in Chapters 8 and 9.

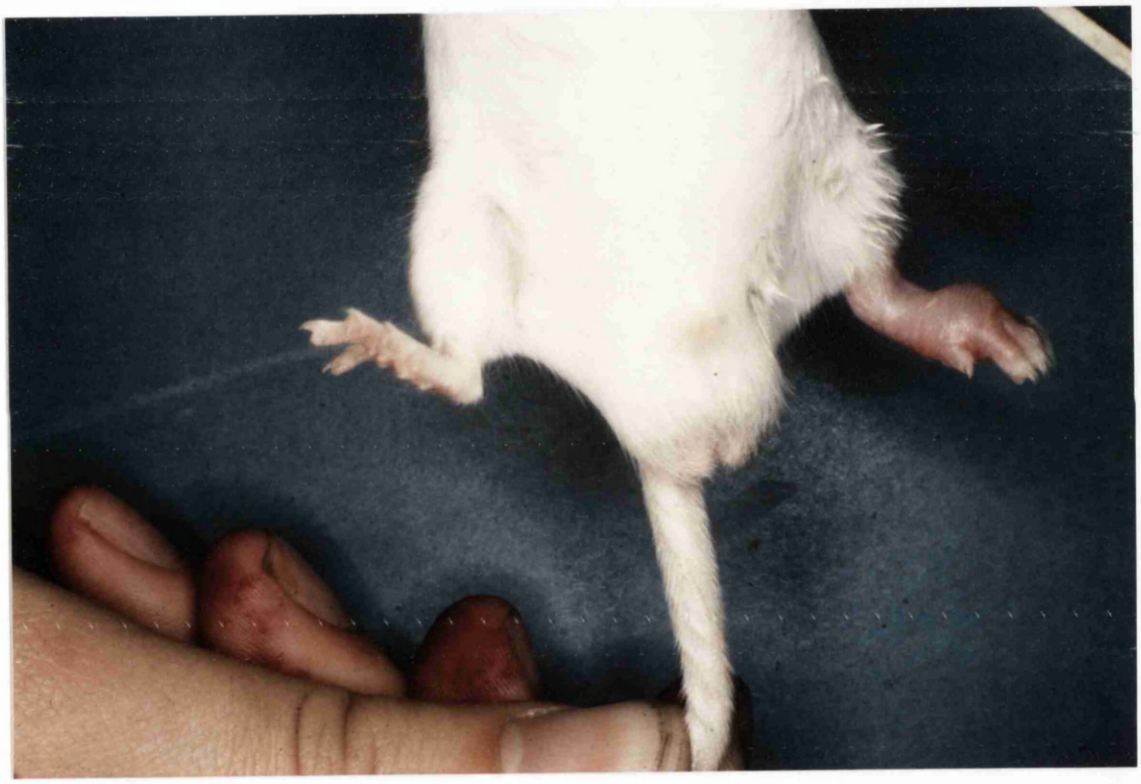


Photo (6): M. ulcerans infection in mouse foot-pad 6 weeks after injection.

Foot-pad size in 1000th of an inch

Progress of oedema in foot-pad infected with M. ULCERANS strain No. 842 Dose (6.7×10^5)

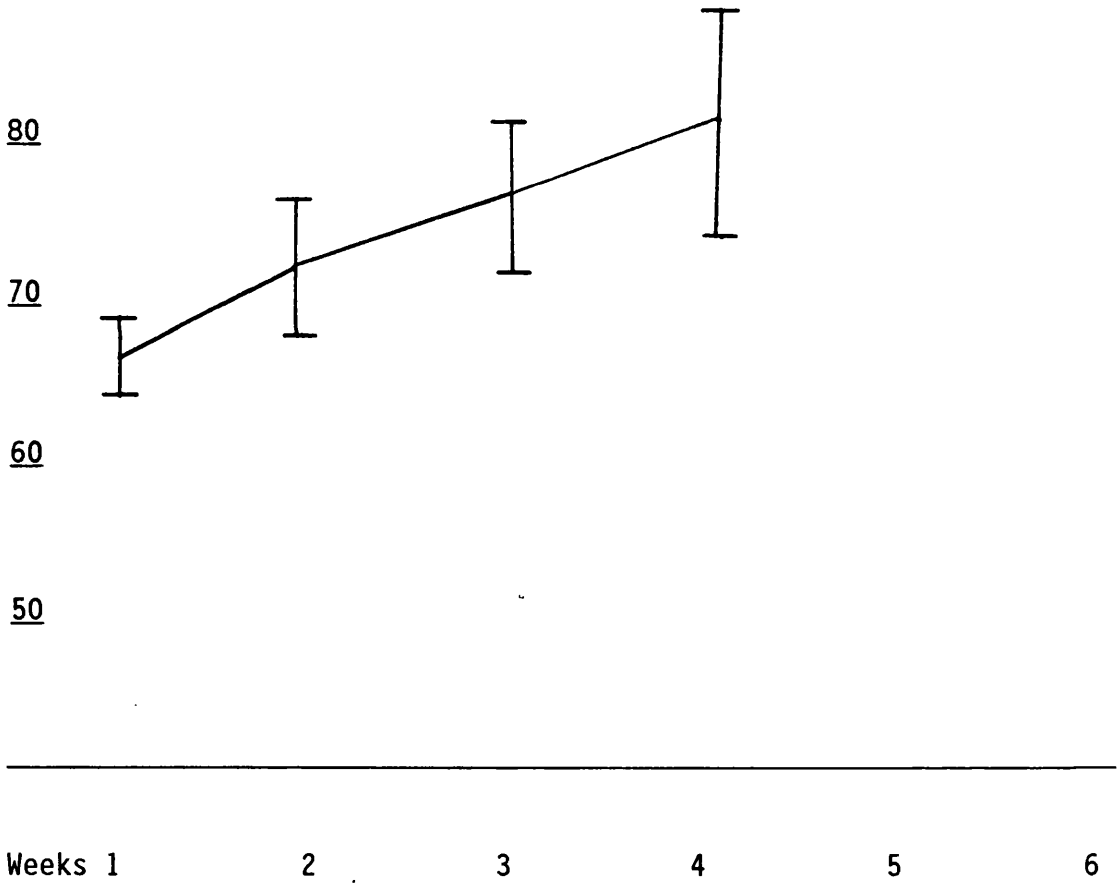


TABLE 4

MICROMETER MEASUREMENTS IN THOUSAND OF AN INCH OF 10 BALB/C FOOT-PADS INFECTED WITH M. ULCERANS

5 mice infected with M. ulcerans and the other five were used as a control

Time When Measurement Taken	Infected Foot Pad		Control Foot Pad		Actual Increase in Size	
		Mean			Infected Foot Pad	Control Foot Pad
Before Infection	67 66 62 64 65	64.8	63 64 62 66 61	63.2	0	0
One Week after infection	70 69 68 65 67	67.8	65 66 63 64 67	65	3	1.8
Four Weeks after infection	72 81 75 77 77 80	77	68 66 63 67 65	65.8	12.2	2.6

D. BIOCHEMICAL TESTING

It is well known to workers with M. ulcerans, that most biochemical tests give negative results. This can be useful in distinguishing M. ulcerans from other slow growing Mycobacterial species with a similar lack of biochemical activity. The species M. haemophilum has been reported as an opportunistic pathogen associated with skin infection and biochemically gives negative results. The chief bacteriological difference between this species and M. ulcerans is the requirement for ferric ammonium citrate in the culture medium (Dawson and Jennis, 1980; Francis and Jean, 1985).

All my cultures of M. ulcerans and a strain of M. kansasii were tested for the following:

1. Catalase
2. Nitrate reduction
3. Cytochemical neutral red reaction

These are the only three reactions that have been reported to be useful in identifying M. ulcerans.

MATERIALS AND METHODS

Methods were taken from C H Collins, J M Grange, M D Yates and Hugh *et al* (1985).

Catalase Test:

- A thick suspension of each culture was made in PBS buffer, total volume of 2ml in 12mm diameter tubes.
- The tubes were heated in a water bath at 68°C for 20 minutes. An equal mixture of hydrogen peroxide (20v/v) and 10% PBS/Tween 80 was prepared and 0.5ml was added to each tube.
- The tubes were allowed to stand for a few minutes at room temperature.
- The height of the column of bubbles was measured in millimetres.
- The only strain producing bubbles was the control organism, M. kansasii.

Nitrate Test:

- A thick suspension of each organism was made in PBS buffer.
- 0.05ml of 4% aqueous potassium nitrate solution was added to each and the tubes were incubated for 4 hours at 32°C.
- The suspension was acidified with a few drops of:
  a) N-hydrochloric acid and 0.05ml of 0.2% sulphanilic acid;
  b) 0.05ml of 0.1ml% 1-naphthyldiethylenediamine-dihydrochloride.
- A pink, positive response was produced by the control organism, M. kansasii. There was no response with the M. ulcerans strains.

Cytochemical Neutral Red Test:

- A loopful of each organism from LJ was inoculated into tubes containing 5ml of 50% methyl-alcohol.
- The tubes were incubated for one hour at 37°C.
- The tubes were centrifuged for 15 minutes at 10 x 100 rpm, and the supernatant discarded. Another 5ml of 50% methyl-alcohol were added and the tubes were incubated at 37°C for a further hour.
- After centrifuging the tubes and discarding the supernatant, 5ml of fresh alkaline buffer was added (1% Na barbitol in 5% NaCl sol.) to each tube.
- 0.2ml of 0.05% aqueous neutral red was added and the tubes were incubated for one hour at 37%, being mixed every 15 minutes.
- Positive results were obtained from M. kansasii with development of a pink to red colour. None of the strains of M. ulcerans produced this colour.

This test was introduced for M. ulcerans by Clancey (1964) who found it in some Ugandan strains.

DISCUSSION

As described in Chapter Two, numerous attempts have been made by several workers in this field to isolate M. ulcerans from the environment and identify its reservoir. Unfortunately none of them succeeded in this task.

The special features of M. ulcerans infection in the mouse are taken as a cardinal characteristic of the species. No other mycobacterium produces the same picture of slow swelling of the injected foot, followed by some ulceration and eventual death of the animal. Although it is not acceptable to allow the disease to progress to its end stages, the swelling and ulceration are quite typical of the disease.

It was with considerable interest therefore, that I found I had three apparently classical strains of M. ulcerans that did not cause progressive disease in the mouse, even when passaged several times. This suggested that these three organisms had lost some particular requirement for causing disease, rather than being merely adapted to an existence on culture media.

During the search for the organism in the environment, it was anticipated that M. ulcerans should cause the classical form of infection in mice, as was always found in the strains isolated from human cases.

The known history of M. ulcerans pathogenicity in man extends four to five decades, since the first case was seen in the late 1930's. Reports of infected cases reached a peak during the late sixties and early seventies when small groups in the tropics were actively looking for cases. Reports declined in numbers as endemic foci dried up. The explanation put forward to explain the changing prevalence that occurred in Uganda and New Guinea was that the floods of the River Nile and Kumusi stream which took place prior to the peak of the incidence had extended the swamps, which were the presumed favoured habitat of M. ulcerans. However, in Australia there is a weaker link between water and infected cases, although an outbreak among people living round the edge of a freshwater lake (Lake Sport) has been reported. (J Hayman 1962)

This observation can be correlated to the possibility that M. ulcerans may be present environmentally as a virulent species of mycobacterium. Barker (1972), carried out one of the largest attempts to isolate M. ulcerans from the environment and he concluded that mycobacteria can be readily isolated from vegetation and soil in buruli areas, but unfortunately M. ulcerans was not recognised among them.

As I have shown, the reliance on the distinctive pattern of response to challenge in mouse foot-pads can be lost in some clinical isolates of M. ulcerans. Moreover, evidence from other studies using other species of mycobacteria showed differences in biochemical activity and antibiotic resistance between clinical and soil isolates. These were attributed to selection of virulent mutant strains of the species

(Grange, 1973, 1974, work on M. fortuitum). This led me to think that M. ulcerans might exist as an avirulent variant in the environment which would not be recognised by the identification based on foot-pad patho-genicity. Thus a few pathogenic variants in the environment might easily be missed except when selected by accidental entry into human tissue.

CHAPTER FIVE

INTRODUCTION

The finding that three strains on M. ulcerans failed to produce the classical pathological features of the organism in mice introduced the possibility that a comparative study of these strains with pathogenic strains might elucidate a virulence factor.

It was important before starting the study to confirm the identity of the non-pathogenic strains as M. ulcerans. Immunodiffusion analysis and DNA probes identifying restriction-fragment-length polymorphisms techniques were used to achieve this. Since the organism is very slow growing, it was difficult to obtain a large number of cells on a protein free medium. Media on which the species might have grown faster all contain proteins or other antigenic substances.

The aim of the next two sections in this chapter is to establish the authenticity of the non-pathogenic strains.

SECTION A: IMMUNODIFFUSION STUDY

INTRODUCTION

Immunodiffusion analysis of bacterial antigens :- Rabbit antisera have been used by many workers to distinguish between mycobacterial species. Notably this system was used to show that strains, under different names, and coming from several countries, all belonged to one species: M. ulcerans (Stanford 1972).

MATERIAL AND METHODS

1. Bacterial Antigen

The strains used for antigen preparation for this part of the study

Strain No.	Pathogenic in mouse foot-pad
750	Pathogenic
7748	Pathogenic
0009	Non-pathogenic

M. kansasii, strain No 1468, Dr J. Stanford collection

These were selected because they grew well in Sauton's medium.
It was difficult to obtain a good amount of cells from strains 00012 and 7833 (non-pathogenic) for use with this technique, but they are used in the next section of the study.

Preparation of the Antigens

The antigen was prepared as described by Stanford (1972). Organisms from fresh culture on Soton's medium were suspended in PBS buffer pH 6.8 and treated for 15 minutes in an MSE 100-watt ultrasonic disintegrator with maximum amplitude. The resulting mixture of residual whole organisms, broken cell wall and cytoplasm was then centrifuged at 10,000 rpm for one hour to remove most of the particles using the MSE high speed 25 centrifuge. The supernatent was filtered serially through 0.45um and 0.22 sterile membrane filters.

Preparation of Rabbit Antisera

A sonicate preparation of M. ulcerans No 1459 (pathogenic strain) was mixed with an equal volume of Freund's incomplete adjuvant (one part of Arlacel A plus nine parts of paraffin) and mixed to a thick consistency by forcing through a 1ml syringe pressed on the conical bottom of a universal container.

One ml volumes of this preparation are injected into alternate flanks of two rabbits at weekly intervals for a total of six weeks. After a further two weeks, the first serum samples were collected from the animals and stored at -20°C. The rabbits were numbered 458 and 459 in the record book. Rabit No. 459 died for an unknown reason a week or two after the initial bleed. Rabbit No. 458 was bled on a number of subsequent occasions.

Preparation of Immunodiffusion Plates

- Sodium azide 10g
- Bacteriological agar (oxoid no. 1) 10g
- Distilled water 1000ml

The mixture was heated to dissolve and dispensed into disposable 98 mm diameter petri dishes, 25ml in each plate. This gives an agar depth of about 3.3 mm. After standing at +4°C for at least 24 hours, discs were cut out of the gel using a cutter producing a central well and six equivalent satellite wells.

Staining the Plates

The plates were soaked in saline overnight to remove non-precipitated antigen, serum and the azide. The gel was removed and placed on a glass and covered with No. 1 filter paper. Holes were poked in the filter paper over each well to prevent bubbles from forming between gel and glass. After drying, a solution of coomassie blue was used to stain the gels.

Staining solution

(0.1255 Coomassie Blue R-250, 50% methanol, 10% acetic acid)

- 2% Coomassie Blue R-250 Solution	62.5ml
- Methanol	250ml
- Acetic acid	50ml
- H_2O	500ml

Decolourising Solution

(7% acetic acid, 5% methanol)

- Acetic acid	700ml
- Methanol	500ml
- H_2O	10.0 litres

THE EXPERIMENT

In each case the serum was placed in the central well and antigens were placed in peripheral wells.

Antigens were arranged so that comparisons could be made between pathogenic and non pathogenic M. ulcerans strains and between M. ulcerans and M. kansasii. Plates were left for approximately one to two weeks at 4^0C for precipitates to form. These were observed by a simple dark ground system and when no further precipitates appeared, the plates were washed, dried and stained as explained above.

SECTION B: EXAMINATION OF DNA RESTRICTION-FRAGMENT-LENGTH POLYMORPHISMS OF PATHOGENIC AND NON-PATHOGENIC M. ULCERANS STRAIN

MATERIALS AND METHODS

The following strains were the subject of study in this particular section of the project.

No. of pathogenic Strains	No. of Non-Pathogenic Strains
7749 0017 0013 0011 0015 7750 842 2097 21789	0009 00012 7833

DNA Extraction

The initial method of DNA extraction was described by R Patel *et al* 1986 and modified by McFadden *et al*, 1987b.

The Method in Detail

- Fresh bacterial strains from an LJ slope were scraped off carefully without
  removing the medium and re-suspended in universal containers of 6-10ml of T.E.N. buffer.

- The universals were placed in water for one hour at 70°C.

- The bacterial suspension was spun for 20 minutes at 10,000g. The supernatent was discarded.

- Protease/Subtilisin Carisberg subtilopeptidase A, alkaline protease type III from Bacillus subtilis (Sigma product) in T.E.N. was added at a final concentration of 10mg/ml. Universals were incubated at 37°C overnight.

- Lysozyme (Sigma product) T.E.N. solution freshly made was added next day to make concentration of 50mg/ml. Universals were incubated at 50°C for six hours.

- The cells were lysed by adding SDS to 1% (WV) and pronase/T.E.N. (Pronase from Streptomyces griseus, Calbiochem product) was added to 3mg/ml. Universals were incubated at 37°C overnight.

- The universals were centrifuged at 30,000g and the supernatent was collected in ependorf tubes. The pellet was saved at -20°C for further use.

- Equal amount of phenol/chloroform and isoamyl alcohol were added to the supernatent and mixed gently. The tubes were centrifuged at high speed in a microcentrifuge for 10 minutes.

- The aqueous phase was collected in another ependorf and the previous step was repeated to ensure DNA-free protein.

- 100% ethanol was added to the ependorf, two volumes to the amount of the supernatent. The tubes were left overnight at +4°C.

- The tubes were centrifuged for 40 minutes in a high speed microcentrifuge, and the 100% ethanol was removed carefully. The DNA was then washed twice with 70% ethanol.

- After the last wash, the pellet was left to dry at room temperature.

- a concentration of 10mg/ml TE/RNAase (sigma product) was added to the pellet. The final volume of the DNA sample was 100ul.

- 2ul of DNA sample was examined in agarose gel to determine the amount of the DNA in the total sample.

Agarose Gel Preparation and Sample Examination

- 1% Agarose (sigma Product) gel was prepared in TAE buffer.

- To each of the 2ul samples, 3ul loading buffer and 8ul H_2O were added.

- The standard sample control was 200ug of DNA previously digested with Hind III restriction endonuclease.

- The agarose gel were placed in a DNA electrophoresis tank (Biotech product) containing TBE (Tris borate electrophoresis) buffer. The samples were loaded in the gel and the gel was exposed to constant electric current (70 volts) for 45 minutes.

- The gel was stained by placing it in a container of ethidium bromide solution (0.5ug/ml) and left rocking for 15 minutes. The DNA was visualised and photographed under ultraviolet light.

The total yield of DNA from the different strains is presented in the following table:

Strain No.	Wt Weight	ng of DNA per 100ul
7749	0.4g	3000ng
0017	0.1g	1000ng
0013	025g	3000ng
0011	0.3g	4000ng
0009	0.15g	2000ng
00012	0.1g	1000ng
7750	0.3g	4000ng
842	0.1g	1500ng
2097	0.2g	3000ng
21789	0.1g	2000ng
0015	0.2g	1500ng

Restriction Endonuclease Digestion

An equivalent of 200ng of DNA from each sample was digested with the PVU II (sigma product) restriction endonuclease according to the maker's instructions. Incubation took place at 37°C overnight.

Separation of the DNA Fragments and Southern Blotting Technique

- The digested samples were loaded in 1% Agarose gel as previously described and run in an electrophoresis tank at 50 volt/per gel strength for three hours.

- A smear of digested DNA was seen under ultraviolet light as an indication of complete digestion. A photograph of the gel was taken.

Blotting technique

- The gel was placed face down on a glass plate covered with No. 2 filter paper. The glass plate was placed horizontally in a tank containing southern transfer buffer.

- Hybridisation transfer membrane was placed on top of the gel (Hybond_N Amersham product).

- Three layers of No. 2 filter paper were placed on top of the hybridisation membrane.

- Another layer of tissue paper was placed on top of the filter papers and levelled with a glass plate. A weight was placed on the glass plate and the whole construction left at room temperature overnight.

HYBRIDISATION OF THE DNA FRAGMENT

The Procedures in Detail

- The hybridisation membrane was washed in 2 x SSC standard saline citrate buffer for 5 minutes and dried.

- The membrane was covered with a plastic film and exposed to ultraviolet light for 3 minutes (the side which was against the gel) to fix the DNA to the membrane.

- The membrane was placed in a plastic box containing 0.1% SDS, 3 x SSC buffer and incubated at 65°C for three hours.

- The 3 x SSC buffer was replaced with hybridisation buffer and incubated for 3 hours.

- A 50ul of the radiolabelled DNA probe was boiled and added to 20-25ml of hybridisation buffer. The previous hybridisation buffer was added to the membrane and placed at 65°C overnight.

- The hybridisation buffer was removed and the membrane was washed twice in 3 x SSC 0.1% SDS buffer at 65% for ½ hour and twice in 1 x SSC 0.1% SDS buffer at 65% for another ½ hour.

- The membrane was autoradiographed for three hours and overnight at -70°C.

- Hybridised probe was removed by treatment with 0.4M sodium hydroxide at 45°C for 30 minutes and then neutralised and the membrane placed at -40°C for further use.

Radiolabelling of DNA Probe and Preparation of the Reaction Mixture

A technique for radiolabelling DNA fragments to high specific activity was described by Andrew Feinberg and Vogelstein, 1984. 35P was used as a label for the DNA probe.

Description of the Method

- The DNA was prepared from a plasmid clone isolated from a genomic library of M. paratuberculosis and was used to distinguish between mycobacterial species isolated from patients with Crohn's disease and other mycobacterial species (McFaddan *et al*, 1987a, 1987).

- A 50ng of DNA probe (PMBR16) was boiled for 5 min and placed on ice.

- The following solutions were added to the probe:
  a) 2.0ul of BSA
  b) 10ul ABS
  c) 1ul of E. coli DNA polymerase enzyme
  d) 2.5ul of label 32p-dcTP

  The final volume is made up to 50ul using d/H_2O and left overnight to achieve maximum incorporation.

  The incorporation of the radiolabel into the DNA probe was checked by preparing 100ul volume of 99ul of 20mM EDTA and 1ul of the radiolabelled probe.

- 10ul of the total volume (100ml) was placed on a glass microfibre filter and left to dry.

- The filter paper placed in scintillation bottles and the incorporation was measured using a liquid scintillation counter.

- The probe was used when it showed more than 50% incorporation of the radiolabel.

Solutions and Buffers Used in the DNA Experiments

T.E. or Tris/EDTA/RNase buffer	10mM Tris Hcl pH 7.5 1mM Na2 EDTA 20ug/mL Rase I
T.E.N. or Tris/EDTA/NaCl buffer	50mM Tris base 50mM NacL 100mM EDTA
T.B.E. or Tris borate electrophoresis buffer	90mM Tris base 90mM boric acid 10mM Na2 EDTA 0.5ug/mL ethidium bromide
Hybridisation buffer	15mL of 20 x SSC buffer 5mL of 100 x Denhard's which consists of 2g ficall, 2g BDA, 2g PVP, 100mL H20) 100ul of Herring sperm DNA boiled for 5 minutes to prevent 10g Dextrim sulphate (Pharmacia product) 1mL of 1M phosphate buffer. The total volume is made up to 100ml with H20 and incubated at 65°C before use
20 x SSC (Standard saline citrate buffer)	3M NaCl 0.3M Na3 citrate
Southern transfer buffer	40mL of 10M NoaH 30mL of 5M NaCl 930mL H20

DISCUSSION OF RESULTS

In 1956 and 1958, Parlett and Yeumans were the first to use immunodiffusion analysis for the differentiation of mycobacteria. Subsequently, this technique was used in taxonomic studies by many workers such as Stanford and Beck (1968); Stanford (1969) and Lind (1960).

In studies by Stanford and his colleagues (reviewed by Stanford, 1973; Stanford and Grange, 1974) double diffusion in agar was used with antigens prepared by rupture of living cells by ultrasonic disintegration. From the analysis of about 1000 strains of mycobacteria, it was found that each species possessed between eleven and fifteen precipitating antigens demonstrable by this method. By comparative analysis using homologous and heterologous strains in each test the antigens are divided into the following four groups:

1. Group one antigens which are common to all species of mycobacteria. These antigens are equivalent to the US-Japan reference antigen 1 and 2, being cell wall polysaccharides arabinogalactans and possibly arabinomannans.

2. Group two antigens which are possessed by all the slow growing mycobacteria. Little is known about the composition of these antigens with the exception of the alpha protein isolated by Yoneda *et al* (1965). This protein was shared by M. avium, M. ulcerans and M. marinum.

3. Group three antigens occur in almost all rapidly growing mycobacteria. No investigative work has been done to identify the biochemical nature of these antigens.

4. Group four antigens which have been defined by this technique are species specific, and they can be protein lipid or polysaccharide in nature. Yoneda *et al* (1965) showed in their work proteins which are specific to given species of mycobacteria. Hunter and Brennan (1981) demonstrated the presence of phenolic glycolipid specific to M. leprae. Misaki and co-workers (1977) have shown an antigenic specificity between the arabinomannans of some species. Raymond *et al* (1985), isolated a structurally and antigenically specific glycolipid antigen specific to Mycobacterium paratuberculosis. Also antigenic specificity was demonstrated serologically for phenolic glycolipid 1 from Mycobacterium leprae in lepromatous leprosy patients on short term chemotherapy and tuberculoid leprosy patients (Cho N. S. *et al*, 1983).

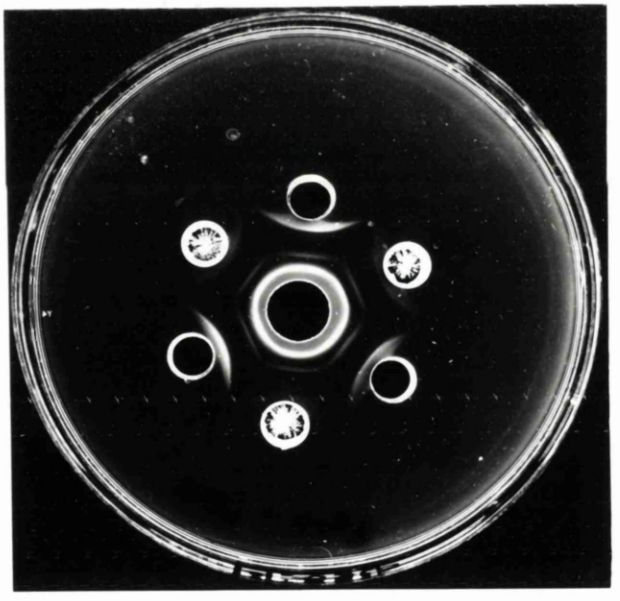
The following Table demonstrates the various antigens of Mycobacterial species arranged in groups.

	Fast Grower Species	Slow Grower Species
Group One Antigens	common antigen	
Group Two Antigens		
Group Three Antigens		
Group Four Antigens	species specific antigen	

The emergence of M. ulcerans as a newly recognised pathogen in several parts of the world required the development of a comparative study between strains isolated from different endemic areas. Such work was carried out by Stanford, 1972. 53 strains from Australia, Malaya, Mexico, Uganda and Zaire were analysed using immunodiffusion tests.

The results showed that M. ulcerans shares five antigens with fast and slow growing mycobacteria, two extra antigens with slow growing mycobacteria and five antigens that are specific to itself.

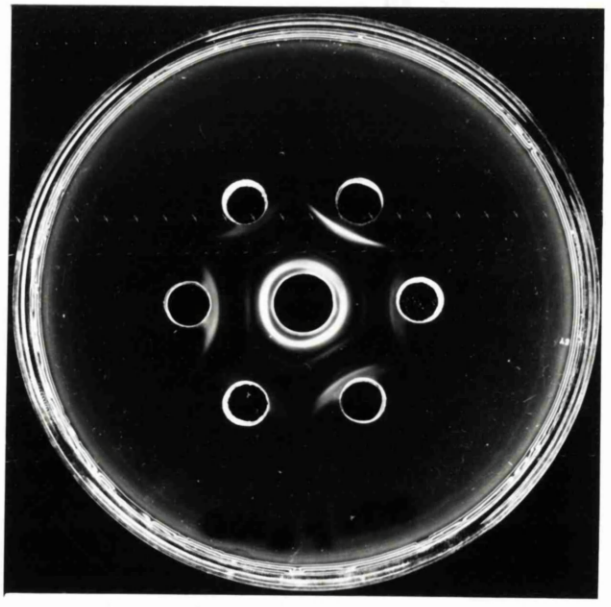
Unfortunately, in my study, I could not demonstrate the full number of antigens described by Stanford, but I was able to show that the non-pathogenic are similar to the pathogenic M. ulcerans. More than four antigens are shared between M. ulcerans strains. Comparison between the pathogenic and non-pathogenic strains showed no difference between the strains. The following photographs are samples from these experiments.



Photograph (1): Precipitation lines of pathogenic strain No. 7748, which are dispensed in the wells which appear white, and non-pathogenic strain 0009 dispensed in the other wells.

The central well contains rabbit 458 antiserum to M. ulcerans strain.

Although there are differences in concentration of individual antigens between the two strains, all have connect around the plate and the strains cannot be distinguished



Photograph (2): Precipitation lines of two pathogenic strains of M. ulcerans No. 7748 and 7750

It can be seen that there is variation between pathogenic strains in relative concentrations of antigens

Using the molecular biological technique, the similarity between the pathogenic and non-pathogenic strains was more evident. The three hour auto-radiograph showed two main restriction sites for PVU II endonuclease which results in 6 and 4 KD DNA bands with the exception of strain 0015, which lacks one PVU II site resulting in the disappearance of 4KD band. Another extra two sites for PVU II enzyme are present in the strains 0017 and 21789. These two sites appeared above the site which resulted in the formation of a 6kDa fragment. Overnight exposure of the autoradiograph revealed more PVU II sites. Above the 0.5kDa marker, all strains have a PVU II restriction site, except for strain 0015. An additional PVU II restriction site appeared in strain 0017 resulting in the appearance of 0.5kDa band. This site is also present in strain 0015.

The results of the auto-radiograph can be summarised as follows:

All species of M. ulcerans including the non-pathogenic strain have three main PVU II restriction sites with the exception of strain 0015 which presents a different picture. An additional two restriction sites were also found within the pathogenic group. A possible explanation for these differences is the geographical origin of the strains. The different restriction sites are represented in the following diagram.

Representation of PVU II restriction sites to various DNA samples from M. ulcerans

Size of DNA Band	9KD	6KD	4KD	2KD	0.5KD
A		|---|	|---|	|---|	
B	|---|				|---|
C		|---|			|---|

A. The common restriction sites found in all species except strain 0015

B. Additional restriction sites were found in strains 0017 and 21789

C. The two main restriction sites found in strain 0015

The bands in this table are a summary of the three hours and overnight auto-radiograph.

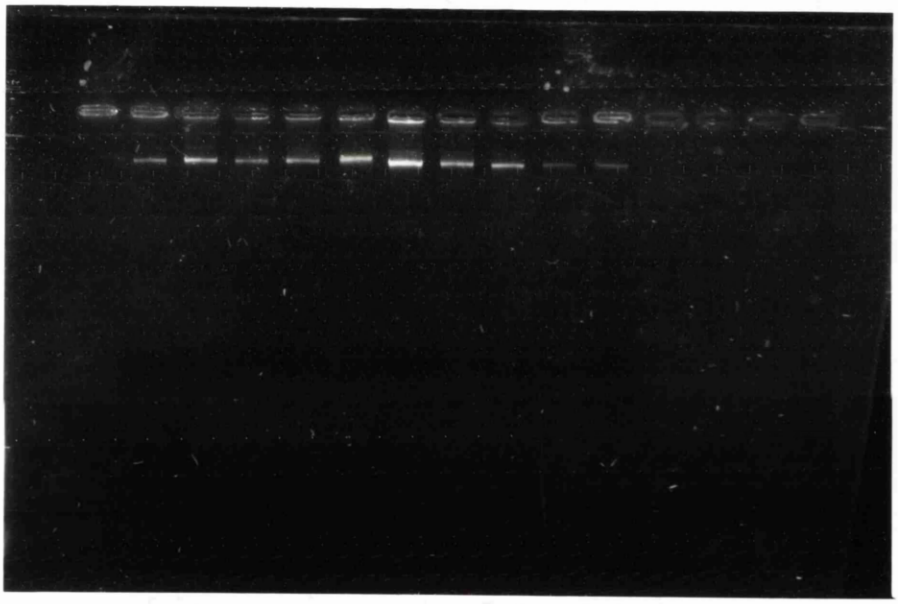
During this stage of the research, I conducted preliminary examination for the possibility that the toxin is encoded by a plasmid or that it might be produced by strains infected with a phage with a gene for toxin production, as is the case in Corynebacterium diptheriae.

Plasmids have been found in several environmentally and clinically isolated species of mycobacterium such as M. avium, M. intracellulare and M. scrofulaceum. The DNA plasmids of these species have been used as epidemiological markers (Messner *et al*, 1986). Most recently, a strong association has been found between increased virulence of M. avium complex isolated from HIV patients and the presence of three

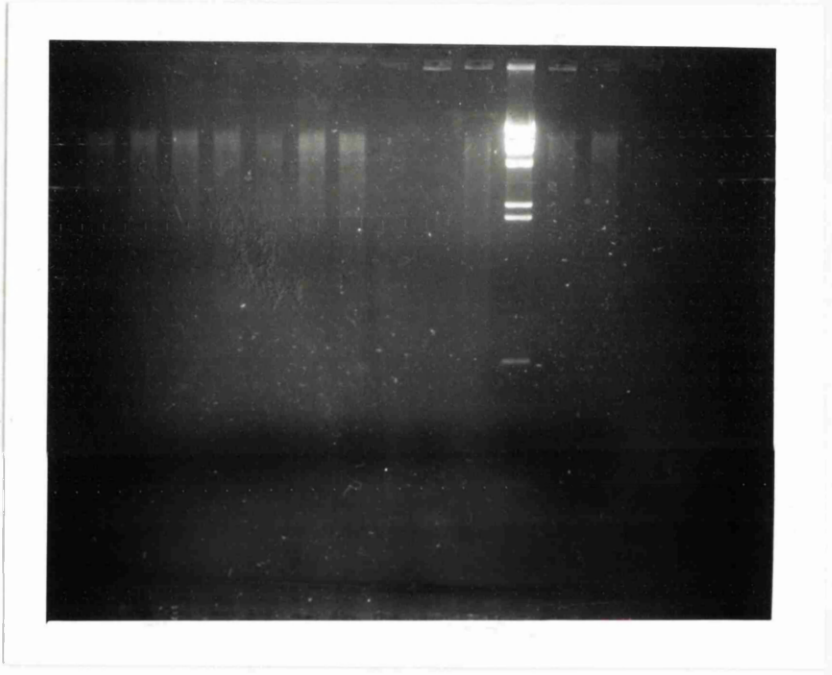
small plasmids. The judgement of this finding was based on the observation of high mortality and increased number of organism in spleen and lung of infected mice (Patticipu *et al*, 1988). Also in another study, M. avium plasmids contributed in the ability of these strains to grow at 43°C and have a higher catalase and peroxidase than plasmid free strain (Pethel *et al*, 1989).

In my case, I couldn't find any plasmids using the whole DNA method of extraction described earlier in this chapter.

With the help of Dr J Grange at the National Heart and Lung Institute, London, I have examined several supernatants of M. ulcerans negatively stained under the electron microscopy and no phages were seen. These two negative results lead to the tentative conclusion that the toxin of M. ulcerans is genetically coded by the chromosomal DNA of the organism and it is unlikely to be secreted by a plasmid DNA.

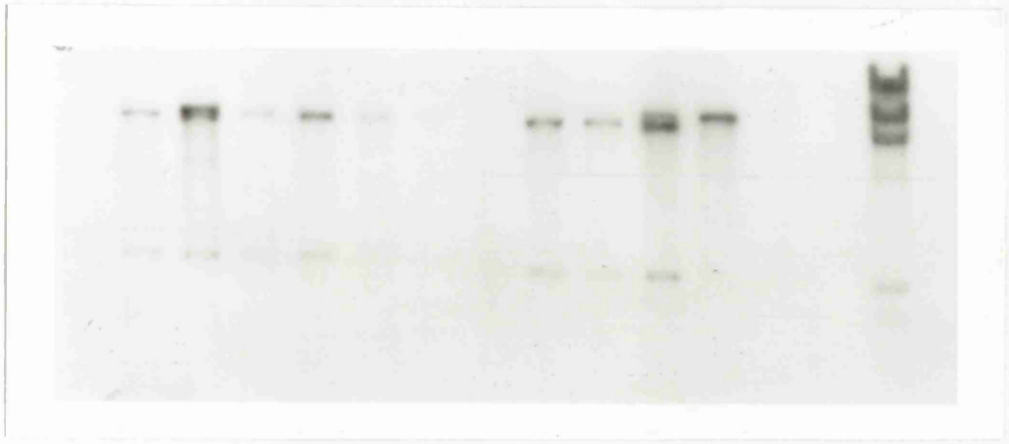


Photograph (3): Example of the DNA extracted from different strains of M. ulcerans

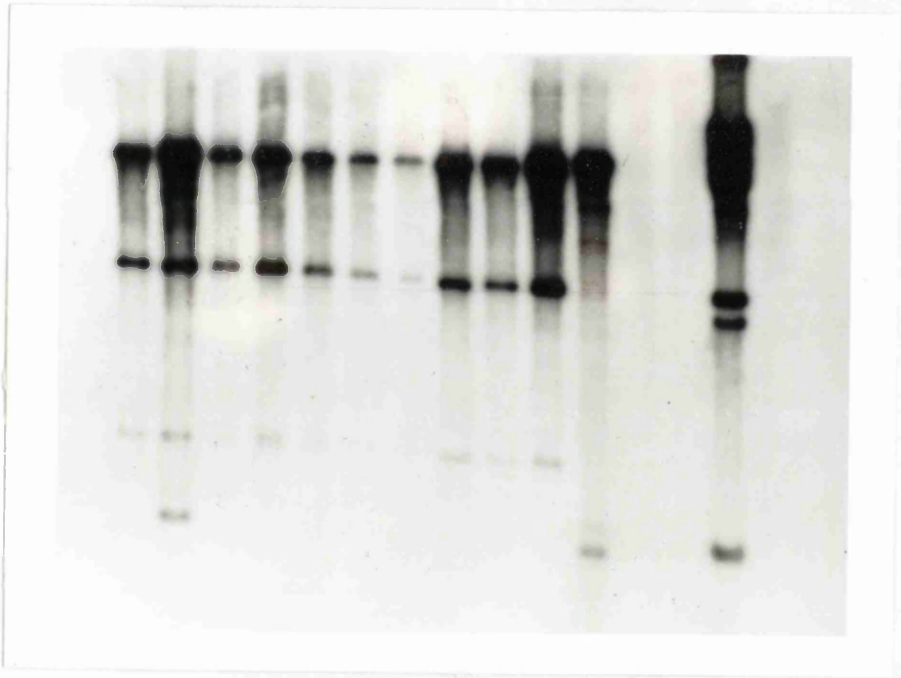


Photograph (4): Example of the digested DNA of M. ulcerans by PVU II restriction endonuclease.

A complete digest of the DNA appears as a clear smear after overnight incubation with the enzyme. The bright bands which appear in this photograph are digest of DNA used as markers.



Photograph (5): Three hours; auto-radiograph of M. ulcerans DNA digested with PVU II restriction endonuclease and probed with PMBR16



Photograph (6): Overnight; auto-radiograph of M. ulcerans DNA digested with PVU II restriction endonuclease and probed with PMBR16

Left to right: Arrangement of strains DNA samples on both autoradiographs 7749, 0017, 0013, 0011, 0009, 00012, 7750, 842, 2097, 21789, 0015 Strain 0009 and 00012 are non pathogenic

CHAPTER SIX

IDENTIFICATION OF TOXICITY IN M. ULCERANS

INTRODUCTION

Bacterial toxins were first recognised as entities by their biological activities. In 1892, Behring and Kitasato were working on toxins of Clostridium tetani and Corynebacterium diphtheriae and produced non-toxic but immunologically active toxoid by heating culture filtrate from these organisms. The idea of using culture filtrates was first introduced by Koch in his attempts to produce a cure for tuberculosis, although Koch failed in his attempts. Later culture filtrates from many species of mycobacteria were shown to contain many products of value in immunological studies on mycobacteria. M. ulcerans is the only species known in the genus to produce a toxin. This was shown in culture filtrates of the organism.

It was interesting to find in a literature search on the toxin of M. ulcerans that all the work regarding it was presented in four papers and carried out by the same group of workers. Moreover, the original association of M. ulcerans with toxicity comes from their evidence (Read JK *et al*, 1974; Krieg *et al*, 1974; Pismler *et al*, 1988; Hockmeyers et al 1978).

In this chapter, I describe an investigation of the toxicity of M. ulcerans, first by looking into different ways of producing the toxin and then at ways of detection of its presence.

METHODS AND MATERIALS

Preparation of Toxins

Slopes of Lowenstein-Jensen medium (LJ) were inoculated with each of the mouse-pathogenic strains, with all three mouse-non-pathogenic strains of M. ulcerans and with three strains of M. kansasii. 2.5mL of sterile RPMI-1640 (Rockwell Park Memorial Institution) medium was added to each slope, and to several un-inoculated slopes, used as blank controls. Cultures were incubated in the upright position at 32°C until good growth of M. ulcerans was obtained (5-6 weeks). (The following is a photograph of the slopes)

Photograph (1): Arrangement from Left to Right
Control LJ slope and inoculated slope

When good growth had been obtained, the liquid medium was pipetted off from each culture including the control slopes, and membrane filtered to 0.2um. These filtrates were the material in which toxin was present. All strains of M. ulcerans were subject to the above method of toxin production and the culture filtrates were tested by the following method of detection.

Detection of the Toxin

The studies on the M. ulcerans toxin linked the toxicity with the pathogenicity of the organism. Since cell mediated immunity plays a major role in the protection against mycobacteria, lymphocyte transformation tests in both human and mouse was used as a relevant system of detection for the toxin. Because M. ulcerans infection is a disease of the skin, skin testing of guinea pigs and mice with different filtrates was used as another system of toxin indication. The guinea pigs used for this purpose were Dunkin Hartley strain, age 42 days and weight 250 grams. The mice used for LTT experiments and skin testing were Balb/c females aged seven to eight weeks old.

Preparation of Mouse Lymphocytes

After the mice were killed by cervical dislocation, they were arranged so the left side faced up to reduce air borne contamination. 70% ethanol was used to wet them completely. A cut was made through the loose skin in the inguinal region, and the peritoneal wall was exposed. 70% ethanol was added to remove any loose hair. By now the spleen

could be seen through the intact peritoneum. A large opening was made in the peritoneal wall over the spleen. The spleen was separated from the vessels and connective tissue, and it was placed in a universal container of 10ml PBS buffer. Gently the spleen was squeezed with forceps to release cells. The cell suspension was transferred into another universal container and the clumps were allowed to settle. The suspended cells were transferred to yet another universal container and centrifuged at 10 x 100 rpm for 8 minutes. The supernatant was discharged and the cells re-suspended in 5mL red cell lysing-buffer (ammonium chloride; 0.829g + KHSO 30.1g dissolved in 100mL d/H_2O. pH 7.4) and left for 5-10 minutes with occasional shaking. The suspension was centrifuged at 200g, the supernatant discharged, and the cells were washed twice with PBS. After the last centrifugation the cells were re-suspended in 10mL RPMI-1640 medium containing 10% FCS. The cells were counted and seeded in a microtitre plate at a final concentration of 5×10^5.

Preparation of Human Lymphocytes

Lymphocytes of a single human donor (myself) were used throughout for the lymphocyte transformation test and later immunological work. 60mL of blood were defibrinated by shaking with glass beads in a glass universal for 10-15 minutes. The blood was centrifuged at 30 x 100 rpm for 10 minutes and serum removed to another universal. RPMI-1640 medium was added to the cells and mixed. An equal amount of lymphocyte separation medium was added to the blood (Flow Lab, Irvine, Ayrshire) and centrifuged for 40 minutes at 17 x 100 rpm. The interface was collected in another universal container and centrifuged for 8 minutes at 17 x 100 rpm.

The supernatant was discharged and the cell pellet was washed twice in RPMI supplemented with 10% autologous serum. The cells were counted and seeded in a microtitre plate at a final concentration of 5×10^5 per well.

Experimental Procedure

Using either mouse of human cells at the concentration described, the final volume of each well in the microtitre plate after adding the culture filtrates was 200ul. The dilutions of the bacterial or control preparations in the cell cultures ranged from 1 in 4 to 1 in 126. All plated were incubated at 37°C in a humid chamber containing 5% CO_2. Adequate stimulation was usually observed by Day 5. Radiolabelling was achieved by adding 20ul of RPMI containing (1uCi) concentrations of (3H) thymidine on Day 5. Cells were harvested on Day 6 by washing with distilled water onto glass microfibre filters using a semi-automated harvester. The filters were left to dry. Each individual disc from each group was separated and placed in glass scintillation vial containing 2mL of scintillant solution. For each individual filtrate tested, seven double dilutions were made, each dilutions effected was measured in 10 wells. A similar 10 wells controls for normal lymphocytes and Con A or BCG activated lymphocytes without adding dilutions of filtrates were made each time the filtrate was tested. The radioactivity was measured as count per minute (cpm) using liquid scintillation counter (1211 Rockbeta) for one minute for each vial.

Initial Experiment

In an initial experiment, the effect of adding 2.5%, 5% or 10% by volume of the filtrate RPMI taken from the different bacterial cultures to Con A or BCG stimulated cells was determined (Table 1 and 2). Also in a separate experiment, dose response of mouse lymphocytes to Con-A and human lymphocytes to Con A and BCG was measured (Figures 1 and 2, Tables 3, 4 and 5).

Table 1. THE EFFECT OF 2.5%, 5%, 10% BY VOLUME CULTURE FILTRATE ON MITOGENICITY OF CON-A ON MOUSE LYMPHOCYTES.

	Inactivated Cells	Activated Cells	2.5% Control	2.5% Test	5% Control	5% Test	10% Control	10% Test
CPM 1	143	2062	2048	73	1999	85	2984	37
2	168	2191	1897	94	2170	79	3830	54
3	180	2236	1785	89	2583	83	3519	68
4	193	2333	2236	71	2917	93	2774	49
5	220	2279	2833	65	2922	76	4169	30
Mean	180	2220	2159	78	2518	83	3695	47
Stimulation Index	-	12	11	0.4	13	0.4	20	0.2

Table 2. EFFECT OF 10% BY VOLUME CULTURE FILTRATE ON BCG DRIVEN TRANSFORMATION OF HUMAN LYMPHOCYTES

	Inactivated	Activated	10% Control	10% Test
CPM 1	223	2825	2332	83
2	201	2731	2400	70
3	220	2757	2587	53
4	192	2799	2510	42
5	180	2618	2436	40
Mean	203	2746	2453	57
Stimulation Index	-	13	12	0.2

Figure 1

DOSE RESPONSE OF MOUSE LYMPHOCYTES TO CON-A MITOGENICITY

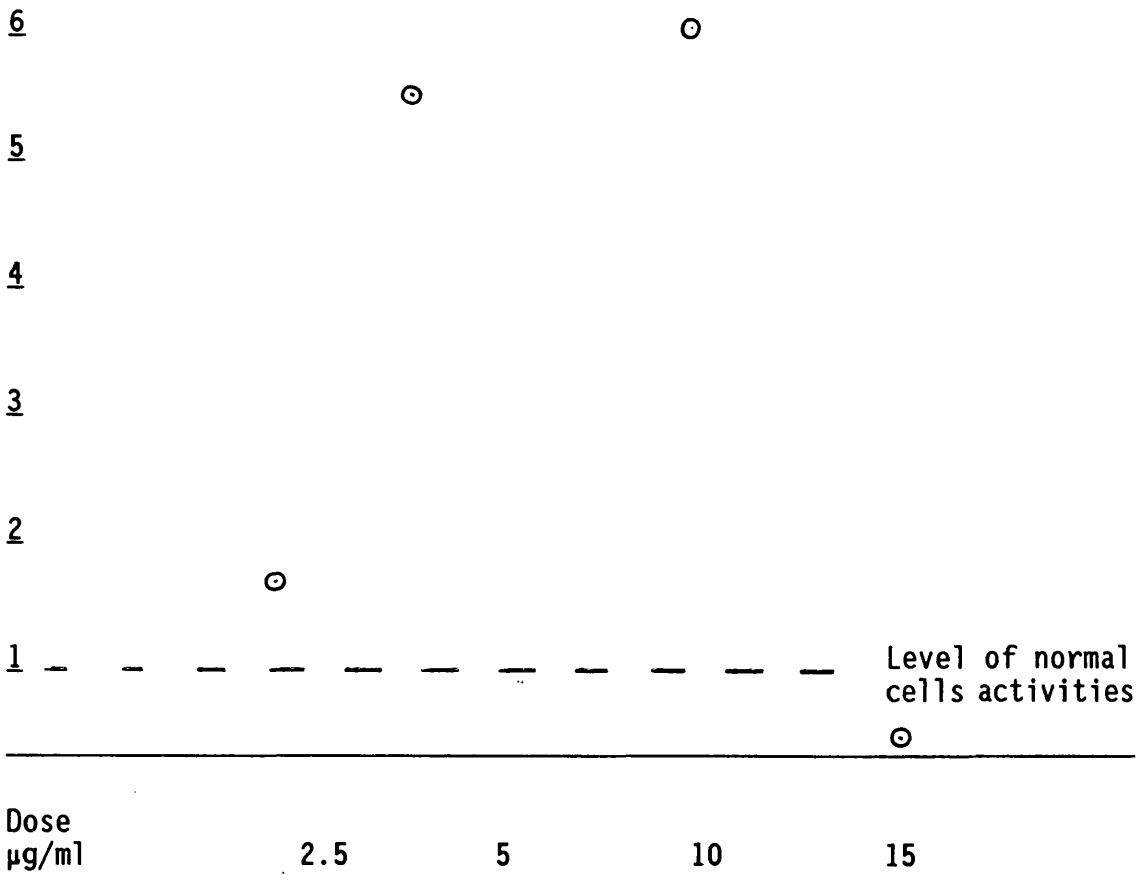


Figure 2

DOSE RESPONSE OF HUMAN LYMPHOCYTES TO BCG ANTIGEN

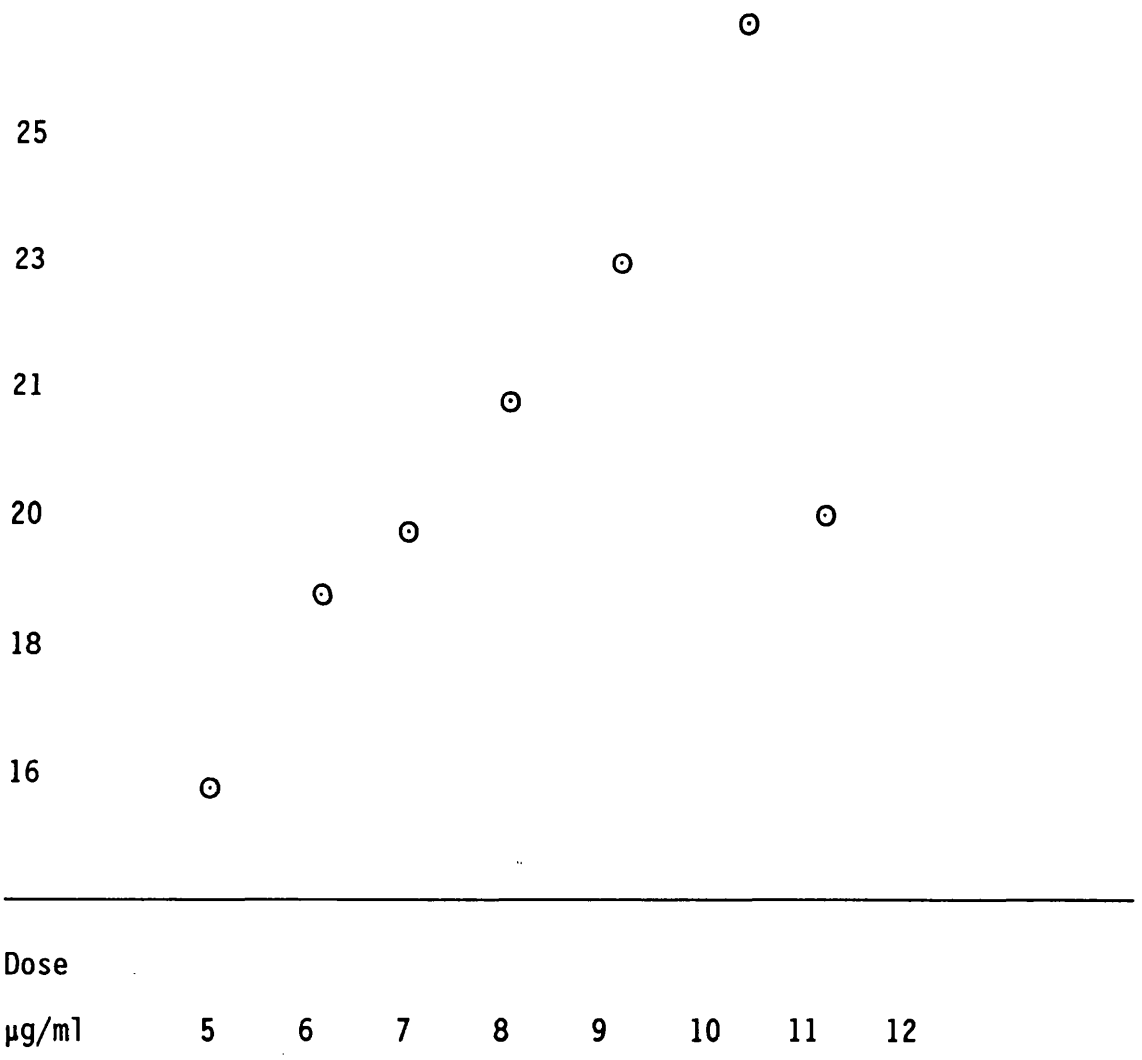


Table 3. DOSE RESPONSE OF HUMAN LYMPHOCYTES TO BCG

BCG µg/ml	0	5	6	7	8	9	10	11
CPM 1	181	2300	3405	3817	3472	4070	4519	2945
2	170	2259	2864	3133	3252	3663	4077	2826
3	173	2909	3436	3043	3231	3675	4032	3216
4	126	2598	3040	2888	3377	3597	3939	3441
5	138	2835	2588	2860	3358	3422	3891	3548
Mean	157	2580	3066	3148	3388	3685	4091	3195
Stimulation Index	-	16	19	20	21	23	26	20

Table 4. DOSE RESPONSE OF HUMAN LYMPHOCYTES TO CON A

Con-A µg/ml	0	1.2	2.5	5	10	15
CPM 1	148	1242	2347	6944	13689	50
2	150	1368	4205	8011	14022	45
3	164	1284	2372	6545	13090	70
4	154	1293	8003	6016	12212	64
5	183	1058	4638	7377	14554	54
Mean	159	1249	4312	6978	13513	56
Stimulation Index	-	7	27	43	84	0.3

RESULTS

Table 5. DOSE RESPONSE OF MOUSE LYMPHOCYTES TO CON A

Con A µg/ml	0	1.2	2.5	5	10	15
CPM 1	123	227	1728	4649	1701	36
2	175	514	1582	2304	1986	30
3	209	656	1836	2710	1620	61
4	852	444	1627	4068	1901	101
5	164	435	1615	3127	1512	83
Mean	304	455	1677	3371	1744	62
Stimulation Index	-	1.4	5.5	11	5.7	0.2

The Results

The results obtained from the initial experiments gave an indication to the concentration required by Con-A and BCG antigen to stimulate the lymphocytes. 5 µg/ml was the optimum dose of Con-A that would give a good stimulation to both human and mouse lymphocytes in the tissue culture system used in this experiment, whereas 7µg/ml BCG antigen is the concentration needed to produce good simulation to both human and mouse lymphocytes.

The 2.5%, 5% and 10% by volume of the toxic filtrates and controls showed the effect which can be induced by the filtrates. The 2.5% of toxic filtrates caused a strong inhibitory effect to activated lymphocytes where as 10% control filtrates indicted that relatively no inhibition can be caused by the controls.

At this stage it is obvious that a titration to the culture filtrates is the next stage.

Culture filtrates taken from slopes inoculated with strains of M. ulcerans known to cause disease in mouse foot-pad, caused suppression to BCG activated and Con A activated human lymphocytes (Table 6 and 7). This effect can be recognised up to 1 : 32 dilutions of the filtrates (Table 6). The control filtrates which were taken from un-inoculated slopes had further stimulation effect on the lymphocytes. This was due to the proteins diffused from the slopes into the liquid media during the period of incubation (Table 8 and 9). The culture filtrates

numbered 0009, 7833 and 00012 did not cause any suppression to Con A activated human lymphocytes (Tables 10, 11, 12). These filtrates were taken from slopes inoculated with M. ulcerans strains known not to cause disease in mouse foot-pad. The culture filtrates taken from M. Kansasii induced higher stimulation than the control filtrates. This is an indication to the antigen secured by this species in culture filtrates (Table 13).

Table 6. DILUTIONS OF TOXIC FILTRATE IN HUMAN LYMPHOCYTES ACTIVATED WITH 5μg/ML CON-A.

Dilution		1:4	1:8	1:16	1:32	1:64	1:128	1:256
CPM	1	28	44	61	47	2154	3754	4417
	2	14	49	96	59	1731	4107	2201
	3	29	37	67	49	2198	3947	3809
	4	40	28	35	53	2219	4476	4055
	5	21	39	51	27	2386	3854	4601
	6	21	39	49	29	1994	3771	4868
	7	14	24	71	42	1358	4313	4514
	8	33	39	53	33	3379	3863	4441
	9	21	35	75	71	2139	3941	4871
	10	40	50	58	30	2379	4077	4713
Mean		22.5	38	61	44	2193	4010	4249
Stimulating Index		0.1	0.2	0.3	0.2	13	25	26

Table 7. DILUTIONS OF TOXIC FILTRATE IN HUMAN LYMPHOCYTES ACTIVATED WITH 7 µg/ML CON-A

Dilution		1:4	1:8	1:16	1:32	1:64	1:128	1:256
CPM	1	31	18	604	4822	6984	3698	5461
	2	82	27	440	4568	5258	3102	4545
	3	20	42	418	2903	7126	4012	5169
	4	45	24	300	3354	5110	2723	3968
	5	40	52	495	5354	4124	4656	4821
	6	31	44	861	5315	6171	4024	4390
	7	28	103	726	4753	5082	6806	4407
	8	29	80	462	3946	7041	3620	4908
	9	33	73	444	3332	5656	3158	3549
	10	17	18	602	7436	3549	4047	3141
Mean		35	48	535	4578	5790	3984	4435
Stimulating Index		0.2	0.3	3	29	36	25	28

Table 8. DILUTIONS OF CONTROL FILTRATE ON HUMAN LYMPHOCYTES ACTIVATED WITH 5µg/ML CON A

Dilutions		1:4	1:8	1:16	1:32	1:64	1:128	1:256
CPM	1	2183	1019	1391	1749	1664	1034	1242
	2	1979	1073	1291	1166	1537	1017	1255
	3	1387	1017	1221	1405	1421	1163	1288
	4	1310	1148	1180	1192	1559	1130	1174
	5	1248	1109	1213	1116	1500	1163	1288
	6	1416	1247	1431	1201	1060	1125	1204
	7	1188	1058	1365	1347	1732	1074	1084
	8	2638	1279	1317	1337	1414	1174	1163
	9	1157	1114	1485	1228	1660	-	1125
	10	1981	1056	1305	1115	1457	-	1130
Mean		1648	1117	1319	1285	1500	1110	1195
Stimulation Index		10	7	8	8	9	7	7

Table 9. DILUTIONS OF CONTROL FILTRATE IN HUMAN LYMPHOCYTES ACTIVATED WITH 7 µg/ML BCG

Dilution		1:4	1:8	1:16	1:32	1:64	1:128	1:256
CPM	1	4307	3653	2408	4307	6611	3613	3308
	2	5977	3375	2428	6340	7112	4603	4473
	3	1084	2005	1295	5977	5577	4482	4822
	4	4953	2644	3354	6499	6364	4132	4568
	5	7374	2470	3534	6938	6042	4724	4521
	6	5124	2823	2220	6229	5225	4339	2903
	7	5522	2032	1587	5563	7158	5352	3354
	8	5641	2902	2136	6524	6492	3437	5706
	9	6059	3481	2423	6295	8476	2815	6255
	10	6492	2453	3291	7408	5641	3051	5354
Mean		5253	2783	2467	6108	6469	4054	4527
Stimulating Index		33	17	15	38	41	25	28

Table 10. DILUTIONS OF CULTURE FILTRATE NO. 0009 - NON PATHOGENIC STRAIN IN HUMAN LYMPHOCYTES ACTIVATED WITH CON-A.

Dilution		1:4	1:8	1:16	1:32	1:64	1:128	1:256
CPM	1	2368	2438	2097	2040	2173	1802	1236
	2	2246	1985	2110	1896	2129	2039	1683
	3	2426	1444	1254	1902	2142	2065	1262
	4	2261	2203	1057	2007	2161	2079	1811
	5	1897	1965	2113	2379	2116	1756	1974
	6	2182	2060	2116	2622	1350	2137	2137
	7	2259	2504	2136	1969	1872	1047	1721
	8	2081	2123	1035	1222	1226	1938	2105
	9	2105	2370	2180	1443	1893	1313	2011
	10	1987	1953	1953	1251	1147	1802	2185
Mean		2181	2104	1805	1873	1820	1797	1821
Stimulating Index		13.8	13.4	11	11	11	11	11

Table 11. DILUTIONS OF CULTURE FILTRATE NO. 7833 - NON PATHOGENIC STRAIN IN HUMAN LYMPHOCYTES ACTIVATED WITH CON-A 5µg/ML.

Dilution		1:4	1:8	1:16	1:32	1:64	1:128	1:256
CPM	1	2616	1878	1875	1877	1803	2861	2143
	2	2116	1996	1880	1967	2091	2810	2272
	3	2063	1660	1738	2064	1836	2949	2431
	4	2224	1589	2104	2295	2272	2428	2062
	5	1588	1533	1981	2018	2379	2730	2184
	6	1496	1732	1973	1927	2379	2762	2014
	7	1593	1697	1880	1784	2371	2546	2104
	8	1635	1872	1838	1889	2950	2800	2064
	9	1831	1747	1920	1816	3123	2885	2258
	10	1591	1633	1858	2377	2899	2544	2204
Mean		1875	1733	1904	2001	2410	2731	2173
Stimulating Index		12	11	12	12	15	17	13

Table 12. DILUTIONS OF CULTURE FILTRATE NO. 00012 - NON PATHOGENIC STRAIN IN HUMAN LYMPHOCYTES ACTIVATED WITH CON-A 5µg/ML.

Dilution		1:4	1:8	1:16	1:32	1:64	1:128	1:256
CPM	1	1595	1836	2575	2825	2249	2347	2313
	2	2667	2199	2133	2788	2174	2603	1948
	3	2241	2049	2409	2574	2340	1877	2081
	4	2591	2395	2443	2229	2301	2242	2296
	5	2000	2224	2496	2040	2672	2700	2237
	6	1827	2089	2415	2131	2196	2074	2653
	7	1836	2089	2415	2131	2196	2074	2653
	8	2089	2353	2589	2026	2234	2298	2244
	9	2050	1950	2610	2185	2161	2203	1909
	10	2025	2532	2782	2203	2357	1819	2160
Mean		2092	2168	2459	2315	2275	2224	2235
Stimulating Index		13	13	15	14	14	14	14

Table 13. DILUTIONS OF CULTURE FILTRATE FROM M. KANSASII - IN HUMAN LYMPHOCYTES ACTIVATED WITH CON-A 5µg/ML.

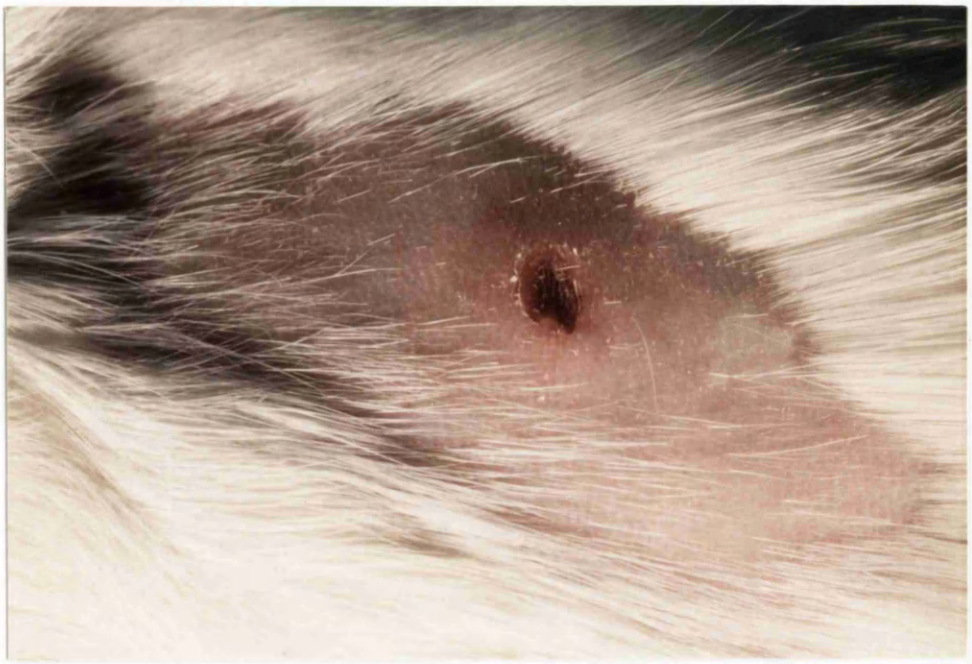
Dilution		1:4	1:8	1:16	1:32	1:64	1:128
CPM	1	2529	1991	2285	2111	2234	2458
	2	2195	2037	2299	2177	1897	2587
	3	2200	2043	2469	2168	2266	2035
	4	2615	1987	2084	2410	2592	2373
	5	2055	2360	2329	2306	2763	2371
	6	2339	2213	2025	2040	2145	2225
	7	2234	2046	2403	2603	2334	2489
	8	1993	1892	2261	2435	3631	2671
	9	1887	2066	2188	2225	1819	2758
	10	21322	1850	2071	3832	1351	2501
Mean		2217	2048	2241	2430	2303	2446
Stimulating Index		14	13	14	15	14	15

Skin Testing of Culture Filtrate

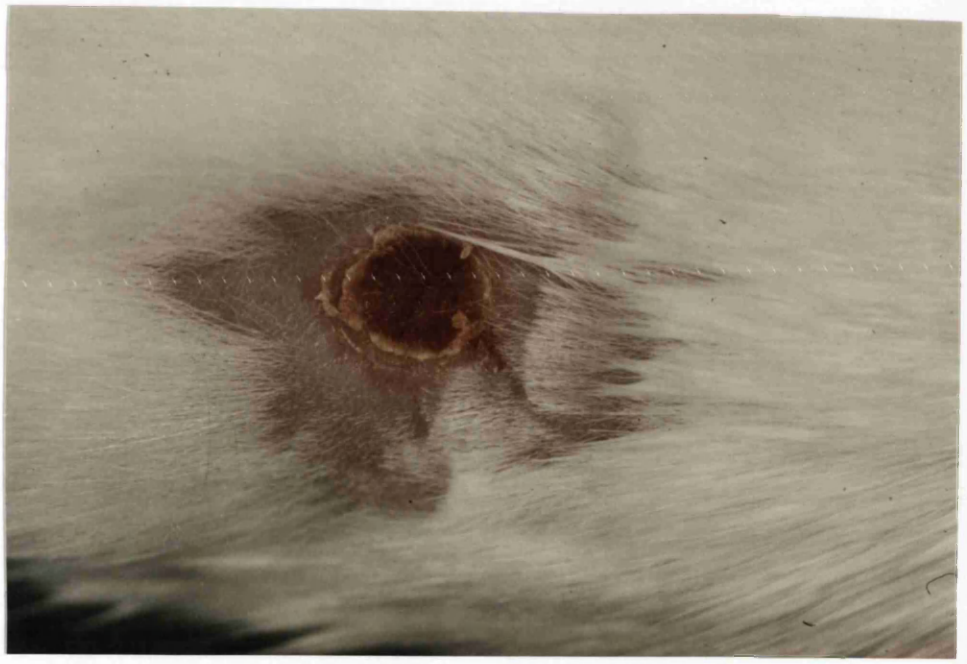
Control suppressive and non-suppressive filtrates of M. ulcerans and M. kansasii filtrates were transferred to dialysis tubing (12000 to 14000 pore size/Medicell International Ltd) and concentrated by dehydration using Aquaside I (Calbiochem). These concentrations of toxin were given by intracutaneous route. One guinea pig and one BALB/C mouse received 0.1mL of suppressive filtrate on one shaved flank and 0.1mL of control filtrate on the other flank, treated the same way. A second guinea pig and a second mouse received 0.1mL of non-suppressive M. ulcerans filtrate on one flank and M. kansasii filtrate on the other flank. A photograph of the effects caused by the toxic filtrate to the animals skin were taken after 48 hours.

The control filtrate did not cause any response in both guinea pigs and mouse. A very slight response caused by the filtrates of M. kansasii and the non-toxic filtrates which disappeared less than 24 hours after the injection given.

The response to the toxic filtrate started approximately 24 hours after the injection and peaked at approximately 48 hours. As the photographs show a severe necrosis is clearly visible. One week after the injection the necrosis healed and the skin appeared normal.



Photograph (2): 48 Hours, Skin Test of M. ulcerans toxic filtrates in non-sensitised guinea pig.



Photograph (3): 48 Hours, Skin Test of M. ulcerans toxic filtrates in non-sensitised BALB/C mouse

Toxicity To Isolated Human Macrophages

In another attempt to demonstrate the toxicity of M. ulcerans, the pathogenic strains were collected from surface of media (L.J. Slope) and a single cell suspension was made in PBS buffer (as described in chapter four). A concentration of 10^5 organism/mL was prepared and 200ul of the suspension was added to human macrophages seeded in a microtitre plate at a concentration of 5 x 10^5 per/well. The macrophages were separated from the population of lymphocytes by allowing the cells to settle in a tissue culture flask for several minutes at 37^0C in a humid chamber. The supernatant was discharged and the adhered macrophages were removed from the surface of the flask by adding 3.3% of EDTA (BDH Product) + autologous serum + RPMI 1640 medium at a ratio of (½ : ½ : 5) respectively. The microtitre plates containing the bacilli and macrophages were incubated at 37^0C in a humid chamber for a week. A number of photographs were taken during the period of incubation at day one, day three and day seven. (Photo Nos: 4, 5 & 6). The results obtained from this experiment were surprising in that at least some of the macrophages survived the seven days of the experiment despite being filled with the Bacilli. This raised the question of toxin activities against the lymphocytes only or the secretion of the toxin is induced in vivo system only.

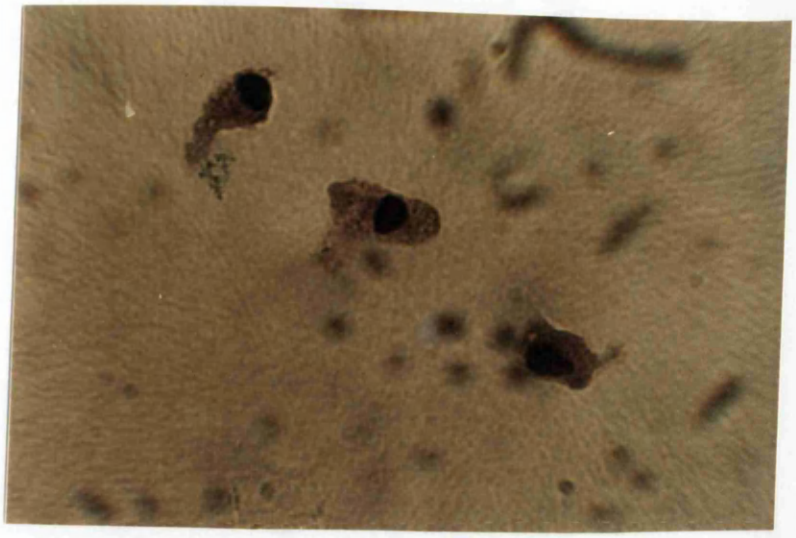


Photo (4): Human Macrophages stained with Ziehl-Neelsen at day one before adding the Bacilli

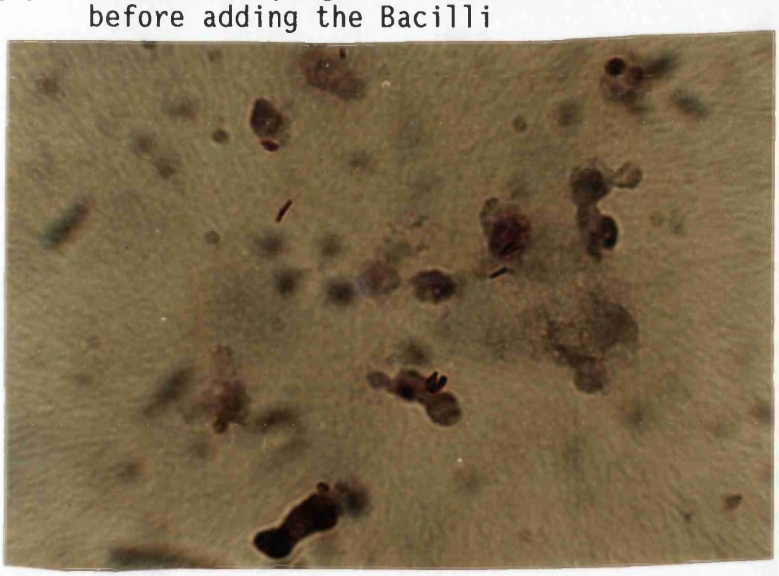


Photo (5): Human macrophages stained with Ziehl-Neelsen at day three. A number of bacilli can be observed attached and inside the macrophages

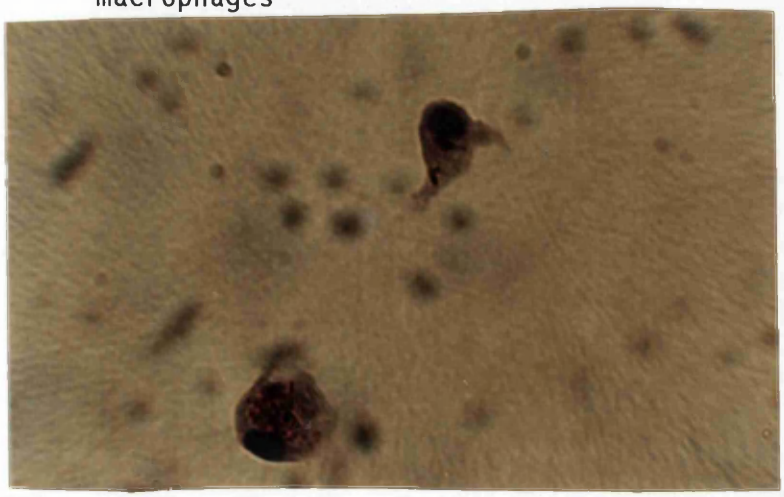


Photo (6): Human macrophages stained with Ziehl-Neelsen at day seven. The macrophages are filled with Bacilli and it is likely that they are still alive

DISCUSSION

Within the mycobacterial genus, M. ulcerans displays a unique character in its culture filtrate. Read *et al*, (1974) and Krieg *et al*, (1974) demonstrated the presence of a cytopathogenic effect on L-929 cells, and Pimsler *et al* (1988) showed *in vitro* suppressive activities on proliferation and phagocytosis of murine spleen cells caused by filtrates of M. ulcerans. These observations were related to histopathological studies of the early lesions caused by M. ulcerans, where large numbers of organisms were observed extracellularly (Connor and Lunn, 1966). My experience has been that growing M. ulcerans in broth media was not successful, although the method used by Read (1974) and Krieg (1974) to produce filtrate of M. ulcerans containing toxic activity was by growing the organism in Dubos broth culture. This may be attributed to the origin of the isolates. The alternative approach I have used achieved similar results but not without disadvantages, such as some ingredients from LJ and Sauton's medium released in the RPMI. In the case of LJ, this created difficulty in measuring the concentration of secreted protein from the organism and later reduced the chance of using a wider range of separation techniques. Nevertheless, these filtrates proved to be indicative of the presence of suppressive activities for both murine and human lymphocytes (Tables 6 & 7). More importantly I established a link between the pathogenicity of the organism and the activities of the culture filtrates.

In the results, the filtrates number 0009, 7833, 00012 and M. kansasii filtrates showed no suppression of the lymphocytes (Tables 10, 11, 12 & 13). Moreover the level of proliferation caused by these filtrates was higher than that caused by the control filtrates which is an indication of the presence of antigenic components secreted by the organisms. Since the control filtrates showed no suppression of lymphocytes, the suppression caused by the suppressive filtrates can only be related to the secreted products of the organism. The stimulation index of the previous results is summarised in Table 14.

Table 14. SUMMARY OF THE STIMULATION INDEX

CONTROL AND TOXIC FILTRATE STIMULATION INDEX'S SUMMARY

Dilutions	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Con-A Group							
Control Filtrate	10	7	8	8	9	7	7
Toxic Filtrate	0.1	0.2	0.3	0.2	13	25	26
Filtrate No. 0009	13	13	11	11	11	11	11
Filtrate No. 00012	13	13	15	14	14	14	14
Filtrate of M. kansasii	14	13	14	15	14	15	-
BCG Group							
Control Filtrate	33	17	15	38	41	25	26
Toxic Filtrate	0.2	0.3	3	29	36	25	28

The filtrates obtained from pathogenic strains varied in their content of the suppressive factor and the toxicity which is demonstrated when the lymphocytes are stained with trypan blue. Strains No. 7748, 7739, 7749 and 7750 appeared to have the highest amount of the toxin produced in a period of 5-6 weeks. This appeared to be associated with their potential pathogenicity observed in mice. The other filtrates obtained from other pathogenic strains caused suppressive activities on the lymphocytes but not as marked as the four strains mentioned above. The difference in the amount of toxin production in culture can be explained by:

a) the amount of culture on the medium surface;

b) the organism must grow in the region where the surface of RPMI comes in touch with the exposed area of LJ medium.

At this stage, I can separate M. ulcerans into two groups:

1. Strains which were able to cause disease in mice and produce suppression of lymphocytes, regardless of the severity of the disease and amount of the toxicity produced in culture filtrate.

2. Strains which lose their ability to induce the disease in mice, simultaneously their culture filtrates do not suppress lymphocyte activation.

An important relationship can be seen between the production of the toxin and the pathogenicity of the strains.

Changing the proliferative factor from Con A to BCG antigen is to eliminate the possibility that Con A might bind to the proteins produced in the media and therefore fail to activate the lymphocytes (Tables 6 and 7). Similar results were obtained when BCG antigen was used to activate lymphocytes. Injection of a small amount of concentrated suppressive filtrates alongside the other filtrates in non-sensitized guinea pigs and BALB/C mice add to the finding that the filtrates not only have suppressive effects on lymphocytes but also cause the death of cutaneous tissue in these animals. The effect seen on the animal skin ranged from severe necrosis to moderate erythema, according to the strain and culture filtrate. Four principal types of polysaccharides, arabinogalactans, arabinomannans, mannans and glucans have been isolated from both mycobacterial culture filtrates and cell wall extracts (Yamamura *et al*, 1965; Misaka and Yukawa, 1966; Azuma *et al* 1967 and 1968; Yamamura, Mikase and Azuma 1972; Misaki, Seto and Azuma, 1974). Mycobacterial mannan and glucan are derived from inner layers of the cell wall (Imaeda, Kanetsuna and Galindo, 1968) and do not elicit immune responses in animals sensitised to whole organisms (Yamamura, Onona and Azuma, 1968). Suppression *in vitro* caused by extracts from mycobacteria other than M. ulcerans have been demonstrated, but a clear differentiation can be established which distinguishes the type of suppression and toxicity caused by M. ulcerans. Lipoarabinomannans have recently been purified from M. leprae and M. tuberculosis (Hunter *et al*, 1986). Both of these carbohydrate-containing substances are highly immunogenic in sensitised animals. In a more specific study, lipoarabinomannan-B of M. leprae induced suppression of T-cell proliferation *in vitro*. The suppressive

effect observed was not due to the toxicity of the antigen, but partly induced by T8+ suppressor cells (Gilla Kaplan *et al*, 1987). The uptake of lipoarabinomannas of both M. leprae and M. tuberculosis by macrophages *in vitro* for approximately 24 hours results in inhibition of IFN-Gamma mediated activation of mouse macrophages (David, 1988). Since the human subject whose lymphocytes were used in these experiments is highly responsive to tuberculin when skin tested, it could be argued that the suppression caused by M. ulcerans filtrates was partly due to secreted antigens such as those described above. If this is the case, and considering that arabinomannan and arabinoglactan are secreted by all species of mycobacteria, all of them would be expected to have the same effect. The filtrates of non-suppressive M. ulcerans strains and M. kansasii did not induce any inhibition of the same lymphocytes, thus if suppression is due to arabinomannans, there is something very special about those of most strains of M. ulcerans. Moreover, these filtrates did not cause damage to the cutaneous tissue of non-sensitised guinea pigs and BALB/C mice. This is an indication that the lymphocytes of these animals did not recognise the common secreted antigen and the damage is caused by a substance which has a direct effect on the cells.

Although the presence of specific lipids can be correlated with the pathogenicity of mycobacteria, so far no understanding of how a particular anatomical or biochemical extracted character of mycobacteria can affect the host in a disease process.

In summary, M. ulcerans toxins can be characterised as:

1. Not an essential constituent of the organism since not all strains have it.
2. Not present in other species, or present in much lower concentration.
3. Actively secreted, hardly present in Sonicates.
4. If an analogue of lipoarabinomannans, they are very special one.

CHAPTER SEVEN

INTRODUCTION

Most of the work associated with the isolation of antigens from mycobacteria has been dictated by the choice of the method of purification and the interest of the individual investigator. This also determined the starting material best suited to their requirements.

The main sources used to isolate mycobacterial products are sterile culture filtrates or extracts of whole mycobacterial cells, but whatever the source of antigen utilised, it has been agreed among workers in this field that antigens should be derived from cultures grown in a totally synthetic medium. Despite the disagreement upon the starting materials best used for the isolation and purification, the difference between contents of cell extracts and culture filtrates has been regarded in the past as quantitative rather than qualitative (Seibert F B, 1949; Seibert F B, Pederson K O and Tiselius A, 1938).

Large efforts were directed towards the isolation of single specific antigens for use in diagnosis of active tuberculosis. Despite all efforts, no specific, readily standardised antigen has been produced for this purpose. Several reasons were given as a justification, amongst which can be considered, the cultural condition of organism, the type of material to be subjected to separation and purification, the method used for separation, and the test system for examining the isolates of the organism themselves.

The methods applied also varied according to the intended use of the antigen. Polyacrylamide gel electrophoresis is a useful analytical tool for discriminating mycobacterial antigens, provided that conditions are standardised. also the resolving power of gel electrophoretic techniques has been greatly improved by the two dimensional precedures. Gels may be stained for proteins and individual components may be identified by their characteristic mobilities. Antigenic components also can be identified in gels by reaction with appropriate antisera.

Physiochemical fractionation methods carry relatively little risk of deactivation and have been used in many attempts to isolate mycobacterial antigens. The methods used included ion-exchange chromatography, molecular exclusion chromatography, and density gradient ultra-centrifugation.

Studies published concerning isolation of mycobacterial products using these methods revealed their complexity. However, none of the ion-exchange chromatographic studies led to the recovery of fractions containing single specific antigens, although a few antigens have been characterised and purified.

Ion exchange chromatography with diethyl-aminoethyl (DEAE) cellulose was used as a fractionation procedure by (Kniker and La Borde 1964, Kniker 1965). Several antigen containing peaks were obtained when culture filtrates of M. tuberculosis and three other mycobacterial species were separated with DEAE. Three major peaks and several minor peaks broadly distributed throughout the eluted fractions were obtained, but species-specific and group related antigens tended to

concentrate in the fraction eluted at high solution concentration.

Similarly, Bennedsen (1970) subjected unheated culture filtrates and cell extracts of M. tuberculosis to chromatography on DEAE-sephadex columns and showed different results from those obtained by Kniker, 1964; 1965.

The method described in the previous chapter demonstrated the presences of toxic and suppressive activities in filtrates of M. ulcerans. Further investigation was needed to characterised these substances.

10% and 7% SDS-polyacylamide gel electophoresis has been tried in order to analyse the contents of these toxic culture filtrates, but this was unsuccessful, mainly because of the presence of high amounts of Albumin which diffused from LJ medium in to the RPMI compartment of the culture. Because the presence of the suppressive activities has to be followed by LTT (described in an earlier chapter), the high performance liquid chromatography (HPLC) LKB system was found to be the most suitable technique for separating the components of M. ulcerans filtrates.

The major difficulties found when this technique was used, were the loss of sample sterility and the reduction of the suppressive activities in the concerned fractions. However, this was avoidable by washing the system with sterile buffer (Azide 0.05% w/v) for as long as

possible, using sterile Eppendorf tubes to collect the fractions, reducing the time of sample exposure to the atmosphere and by concentrating the original sample by freeze drying before use.

MATERIAL AND METHODS

Five culture filtrates were tested after fractionation to demonstrate the presence of suppressive activities. Three filtrates were obtained from pathogenic strains, one from a non-pathogenic strain and one from a control culture filtrate (uninoculated slopes).

The culture filtrates used in the experiments of this chapter

Toxic Culture Filtrates	A) 7634 B) 0013 C) 0017
Non Toxic Culture Filtrates	A) 0009
Control Culture Filtrates =	Uninoculated slopes

Each sample was fractionated by the HPLC-LKB system on a silica gel column (Tsk-3000, SW size, 7.5 x 600mm Pharmacia product). The pressure of the system was measured at 9 to 10 Bar. The absorbance was measured at 280nm with an LKB 2158 Uvicord-module (Pharmacia product). The scale of the absorbance was recorded at O.D, 0.5/20cm using an LKB-2210 recorder (Pharmacia product). The whole system was controlled by a control unit 2152, HPLC-LKB (Pharmacia product). The buffer used for running the system was phosphate buffer.

Preparation of Phosphate buffer:-

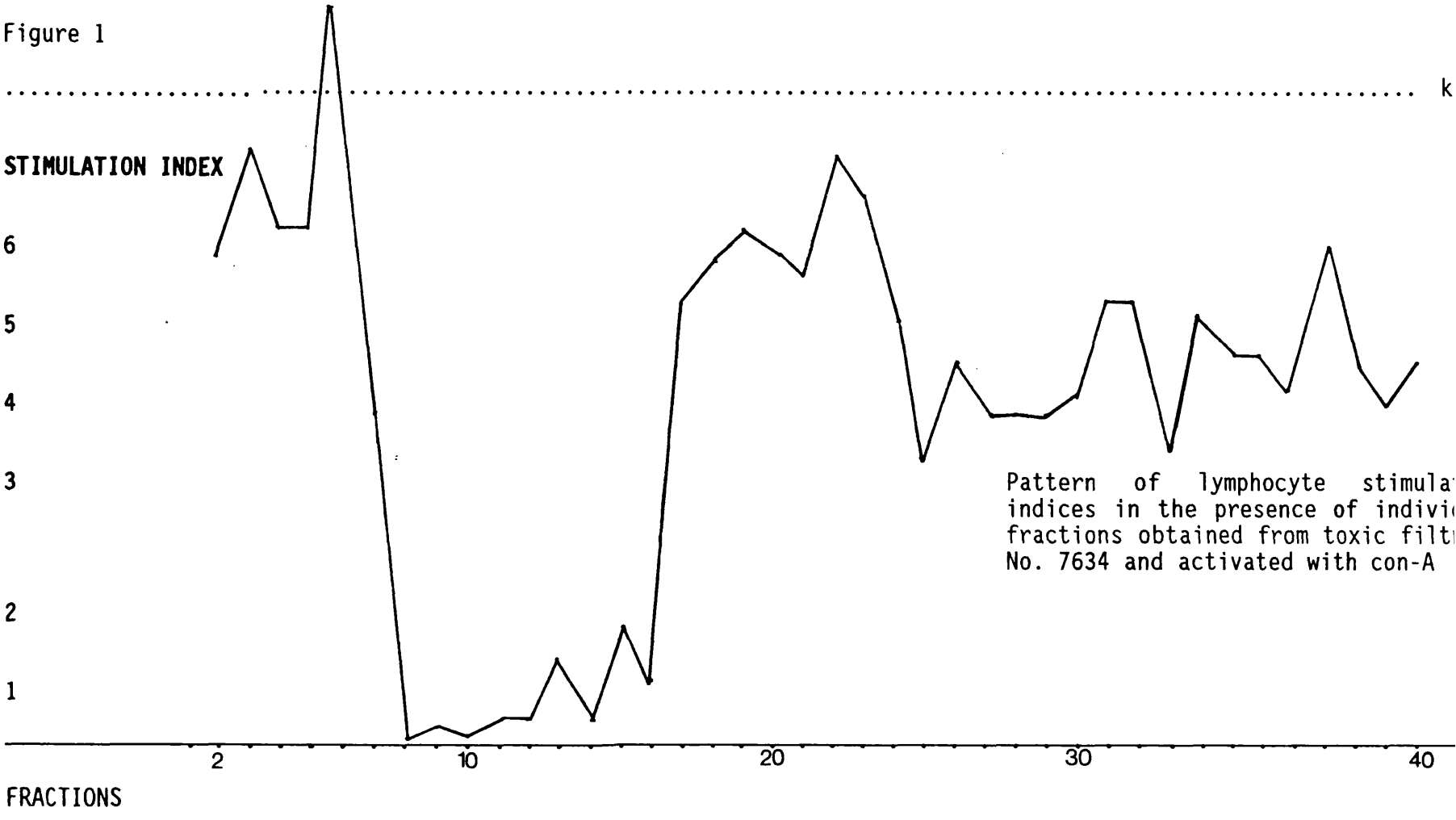
NaCl/8g
KH_2PO_4/0.2g
NA_2HPO_4/1.135g
KCl/0.2g
One litre distilled H_2O

All fractions obtained from filtrate numbers 7634 (toxic) and 0009 (non-toxic) were tested in LTT. Only selected fractions from other samples were tested. The starting volume injected into the HPLC circuit was 200ul and the final volume obtained from each of 40 fractions was 0.5mL. Four wells of con-A activated human lymphocytes were used to test each fraction, 100ul of each fraction was used for each well. Gel filtration molecular weight marker from Sigma product No NW-GF-1000 was used to determine the molecular weights of the fractions obtained from culture filtrates.

Results:-

Stimulation of human lymphocytes by Con A in the presence of fractions obtained from three toxic filtrates (7634, 0013, 0017) showed various responses. The fractions number 8, 9 and 10 (Tables 1A, 1B, 4A and 5A) appeared to cause suppression to the activated cells. Similar fractions obtained from the control filtrates and non-toxic filtrates (0009) did not induce any suppression to the lymphocytes (Tables 2A, 2B and 3A).

Figure 1



Pattern of lymphocyte stimula
indices in the presence of indivi
fractions obtained from toxic filt
No. 7634 and activated with con-A

TABLE 1A: LTT RESULTS OF TOXIC FILTRATES OF NO. 7634 AFTER FRACTIONATION

Fraction No.	2	3	4	5	6	7	8
CPM	- 6247 6688 6198	5189 8614 - 8979	6129 6005 6984 6984	5643 5527 8613 -	4145 9589 6417 6377	4731 4359 4955 -	34 35 143 76
Mean	6377	7594	6525	6595	5681	4681	72
Stimulation Index	35	41	36	36	61	25	0.1

TABLE 1B

Fraction No.	9	10	11	12	13	14	15
CPM	53 126 89 325	38 105 25 44	378 764 1863 1676	1039 1131 1031 1176	1002 7626 1077 1044	1097 1096 1048 1155	1222 1022 1715 7704
Mean	148	53	1170	1099	2687	1099	2924
Stimulation Index	0.8	0.2	6.4	6	14	6	16

TABLE 1C

Fraction No.	16	17	18	19	20	21	22
CPM	1109 1149 3316 4331	1095 9098 6998 7023	7431 4771 6354 6796	9204 6135 5108 6732	5721 4708 8554 6360	8555 6408 4055 5022	9441 6559 6935 7136
Mean	2476	6053	6338	6794	6335	6010	7517
Stimulation Index	13	33	35	37	35	33	41

TABLE 1D

Fraction No.	23	24	25	26	27	28	29
CPM	- 7017 6437 6302	9253 4601 4078 4681	482 4568 4521 2903	3354 5706 6265 5354	5315 4753 3481 3946	5315 4746 4321 3332	6037 3842 4034 4021
Mean	6585	5653	4203	5169	4373	4428	4483
Stimulation Index	36	31	23	28	24	24	24

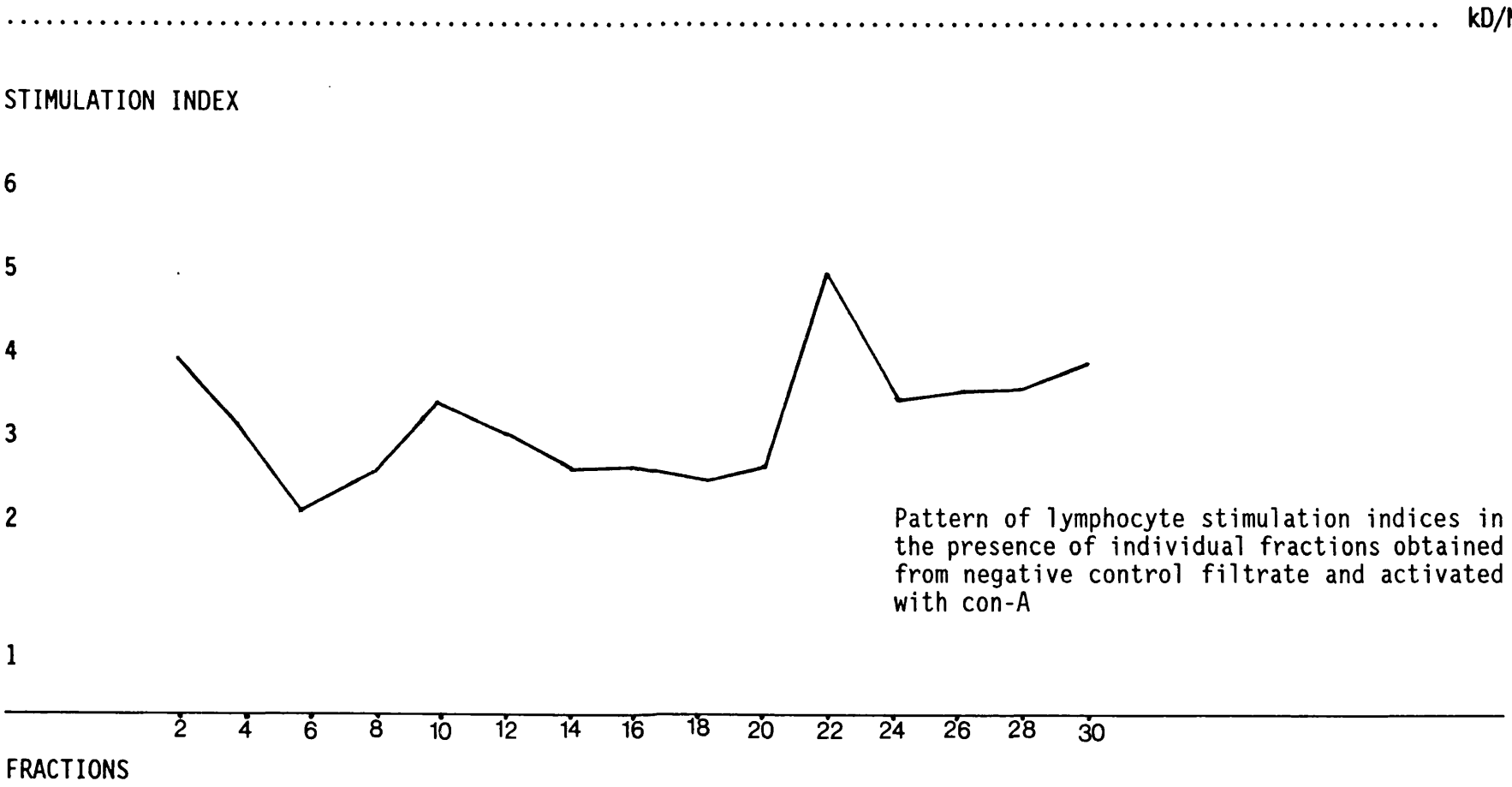
TABLE 1E

Fraction No.	30	31	32	33	34	35	36
CPM	6457 3750 3671 4558	5310 5549 8251 5342	7873 6335 5298 4693	5975 4566 5044 4235	7532 5333 5588 4117	7436 4854 4911 4843	3380 3462 5936 4659
Mean	4609	6113	6049	4955	5647	5511	4359
Stimulation Index	25	33	33	27	31	30	24

TABLE 1F

Fraction No.	37	38	39	40
CPM	8989 5636 5465 6062	- 6289 5453 4421	- 5447 4575 4172	5582 5284 - 4926
Mean	6538	5387	4731	5264
Stimulation Index	36	29	26	29

Figure 2



Pattern of lymphocyte stimulation indices in the presence of individual fractions obtained from negative control filtrate and activated with con-A

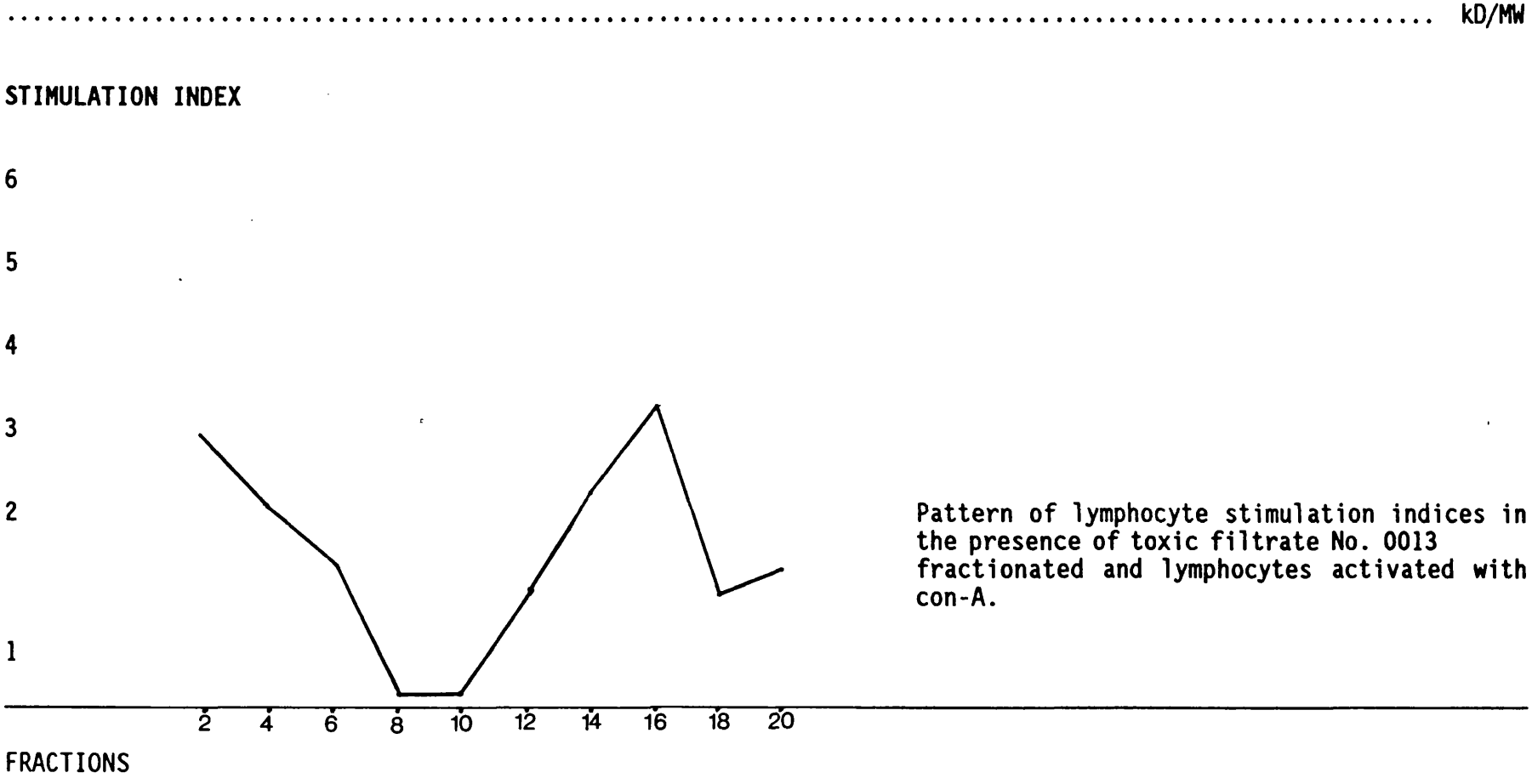
TABLE 3A: LTT RESULTS OF NEGATIVE CONTROL FILTRATE SAMPLES AFTER FRACTIONATION (UNINOCULATED SLOPES)

Fraction No.	2	4	6	8	10	12	14	16
CPM	4763 3273 3082 1559	1825 3498 2769 2260	2892 2596 1066 3039	2326 2055 1721 1946	2711 2632 2662 -	2038 1729 4410 1603	1681 4457 1514 1212	3467 2418 1200 -
Mean	3169	2588	1675	2012	2668	2445	2216	2361
Stimulation Index	4	3.2	2	2.5	3.3	3	2.8	2.9

TABLE 3B

Fraction No.	18	20	22	24	26	28	30
CPM	1493 3365 2400 1525	4574 3544 4506 3352	2340 3459 3195 2865	1215 2064 3564 4067	3071 3510 3315 1233	2545 2316 3299 3395	- 4038 3985 1842
Mean	2195	3994	2964	2727	2782	2888	3288
Stimulation Index	2.8	2.9	2.7	5	3.5	3.6	4.1

Figure 3



Pattern of lymphocyte stimulation indices in the presence of toxic filtrate No. 0013 fractionated and lymphocytes activated with con-A.

TABLE 4A: LTT RESULTS OF FILTRATES NO. 0013 AFTER FRACTIONATION

Fraction No.	2	4	6	8	10	12	14	16
CPM	3670 3110 1435 1447	1814 1660 1545 -	1438 1655 1285 1362	567 179 189 -	190 152 178 134	1661 1190 1166 1241	1419 1239 2276 2706	2472 2421 3199 -
Mean	24155	1643	1435	311	163	1314	1910	2697
Stimulation Index	3	2.1	1.8	0.3	0.2	1.6	2.4	3.4

TABLE 4B

Fraction No.	18	20
CPM	1029 1348 1460 1376	1502 1280 1334 1294
Mean	1303	1352
Stimulation Index	1.6	1.7

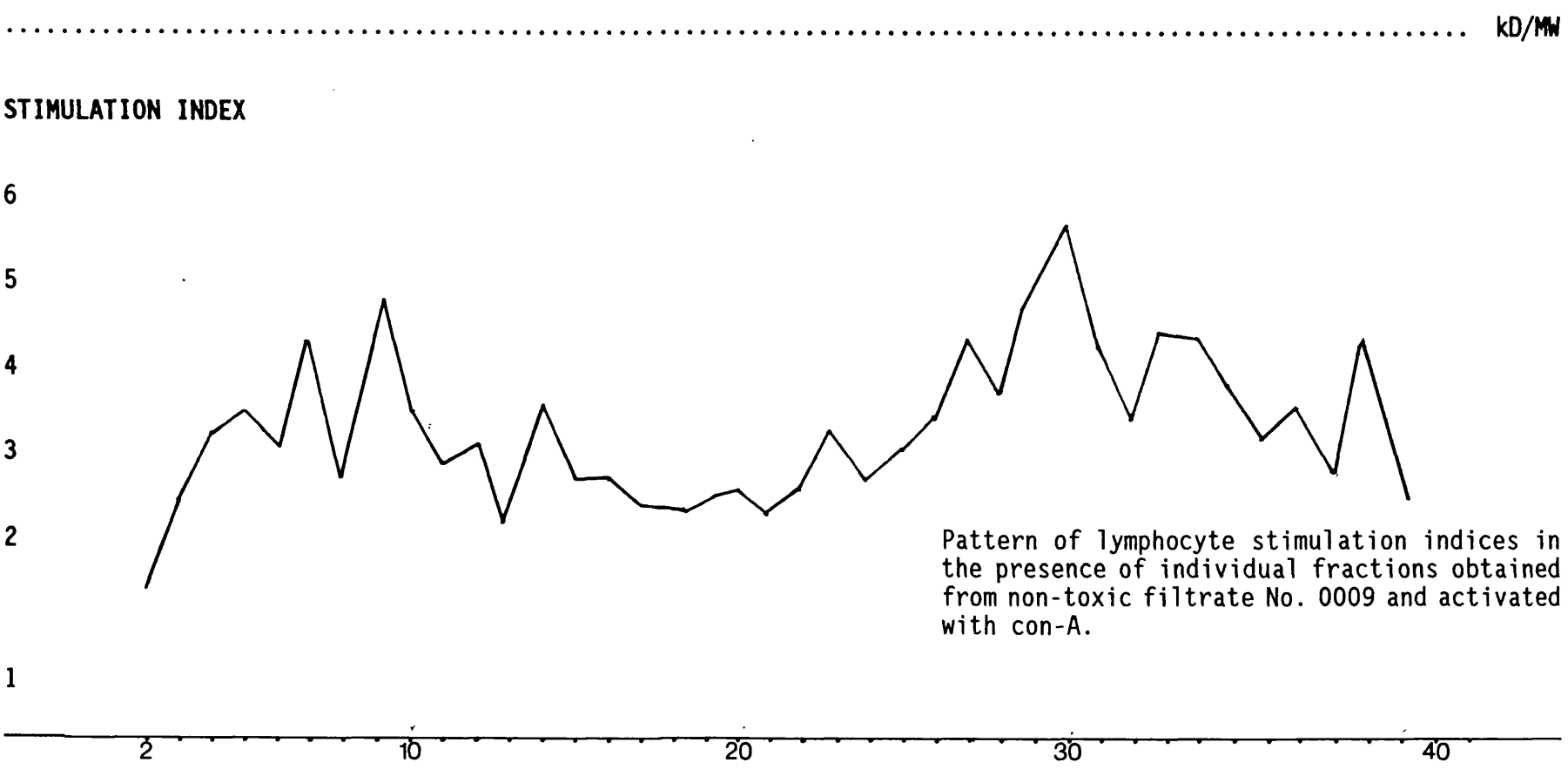
TABLE 5A: LTT RESULTS OF FILTRATE NO. 0017 AFTER FRACTIONATION

Fraction No.	2	4	6	8	10	12
CPM	1479 1293 1473 1502	4179 1365 2947 1031	1801 3181 3083 -	979 793 893 -	1270 1010 973 1147	2870 2976 2474 1450
Mean	1436	2380	2688	888	1100	2442
Stimulation Index	1.8	3	3.4	1.1	1.3	3

TABLE 5B

Fraction No.	14	16	18	20
CPM	2566 2283 2936 -	2069 3177 3057 3242	3131 2118 3707 2387	2504 2408 1987 3878
Mean	2595	2886	2835	2694
Stimulation Index	3.2	3.6	3.5	3.4

Figure 4



Pattern of lymphocyte stimulation indices in the presence of individual fractions obtained from non-toxic filtrate No. 0009 and activated with con-A.

TABLE 2A: LTT RESULTS OF FILTRATE NO. 0009 AFTER FRACTIONATION

Fraction No.	2	3	4	5	6	7	8	9
CPM	1289 1385 1525 1400	1365 1493 3200 1418	1467 3212 3514 2457	1681 - 2441 4329	4662 1632 2711 1946	4268 5668 1401 2363	3275 2371 1294 2334	3280 2502 4374 4381
Mean	1399	2199	2662	2817	2737	3425	2318	3634
Stimulation Index	1.7	2.6	3.3	3.5	3.4	4.3	2.9	4.6

TABLE 2B

Fraction No.	10	11	12	13	14	15	16	17
CPM	4384 2384 3358 1376	4372 1260 1348 -	2421 1472 3239 2419	2135 2242 1149 -	1283 4714 3251 1162	- 2186 3176 1277	1121 2262 4132 1386	1298 2364 2386 -
Mean	2878	2326	2387	1842	2602	2213	2225	2016
Stimulation Index	3.6	2.9	3	2.3	3.2	2.8	2.8	2.5

TABLE 2C

Fraction No.	18	19	20	21	22	23	24	25
CPM	2386 1318 3484 1358	1332 1332 3277 2397	1548 1548 3422 1434	- - 1545 1676	1454 1454 2443 1362	1551 1551 2548 -	1484 1484 2676 -	2596 3066 1826 -
Mean	2136	2094	2239	1901	2195	2550	2233	2496
Stimulation Index	2.7	2.6	2.8	2.4	2.7	3.2	2.8	3.1

TABLE 2D

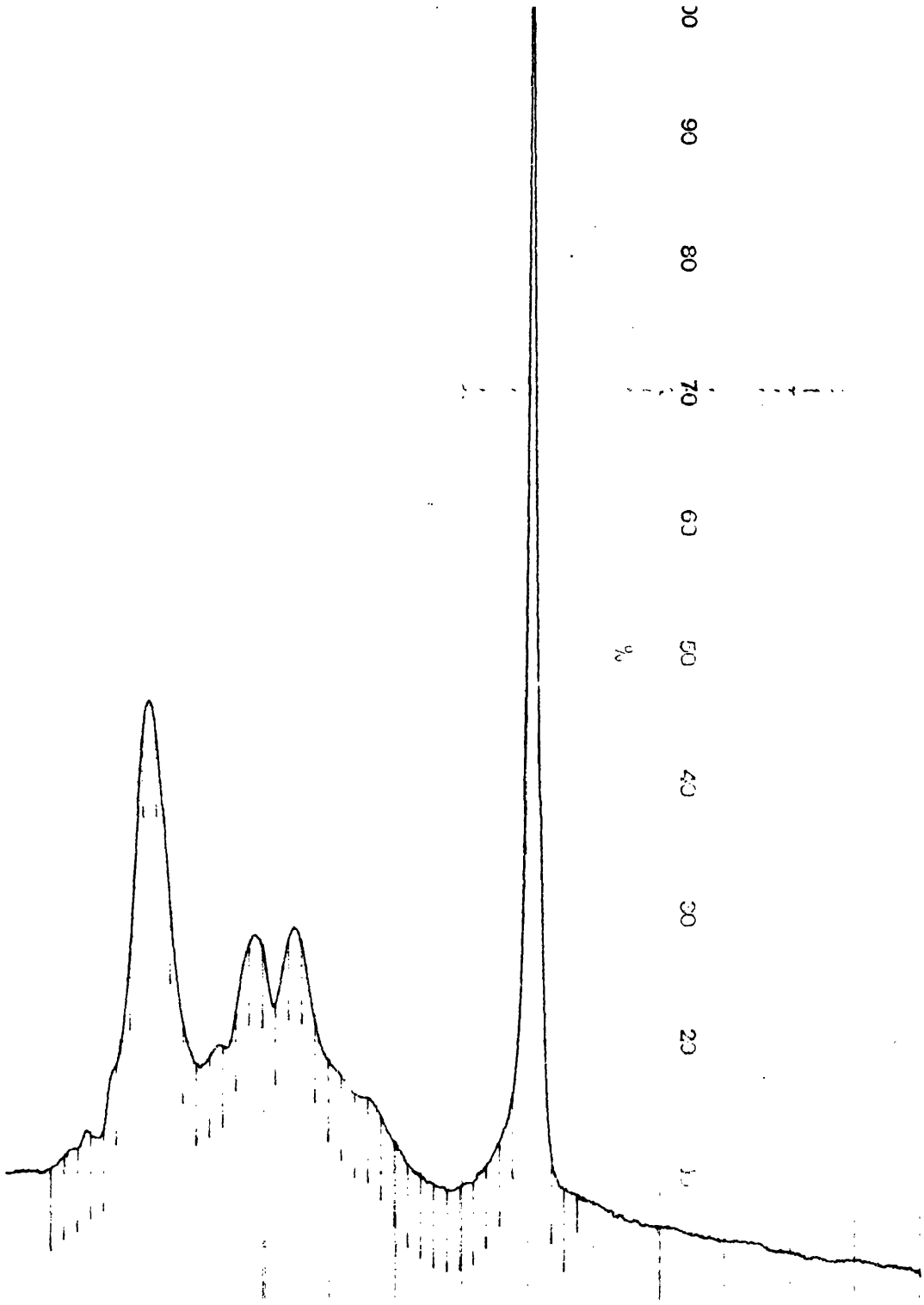
Fraction No.	26	27	28	29	30	31	32	33
CPM	2825 2726 2979 1979	3350 4267 5137 1070	1179 5398 1172 4406	3495 3952 4364 -	4082 4007 5136 -	3395 3299 3110 -	3315 3510 3071 1067	2564 3215 4865 3095
Mean	2627	3456	3039	3937	4408	3268	2740	3434
Stimulation Index	3.3	4.3	3.8	4.9	5.5	4.1	3.4	4.3

TABLE 2E

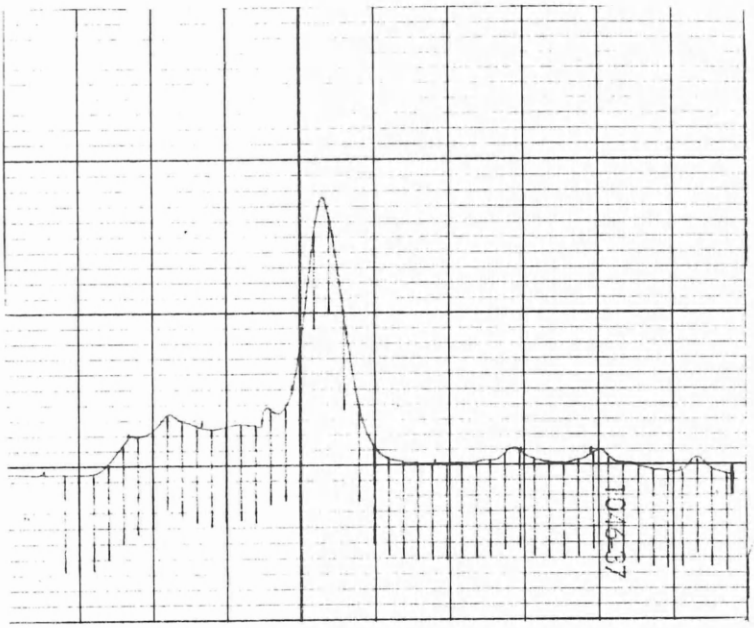
Fraction No.	34	35	36	37	38	39	40
CPM	3340 2352 4506 3544	4231 2870 2976 2474	2450 2566 - 2283	3069 1377 3242 3131	1118 2707 2387 2708	3519 2767 4128 -	1293 2043 2751 2698
	3435	3137	2433	2704	2230	3471	2916
Mean	3435	3137	2433	2704	2230	3471	
Stimulation Index	4.3	3.9	3	3.4	2.8	4.3	2.7

The behaviour of other fractions in LTT varied, some appeared to induce stronger stimulation than control fractions observed from Figs. 1, 2, 3 and 4. The variation of these responses may be due to antigen secreted by the organism or the diffused proteins from L J medium. The results are actually very striking and show that active suppression of human lymphocyte responding to the mitogen Con-A is suppressed by a substance produced by pathogenic strains of M. ulcerans which is missing from the non-pathogenic strains and from control extracts of the bacterial culture medium. That this substance is likely to be the same in each case is indicated by its appearance in the same fraction number in each preparation.

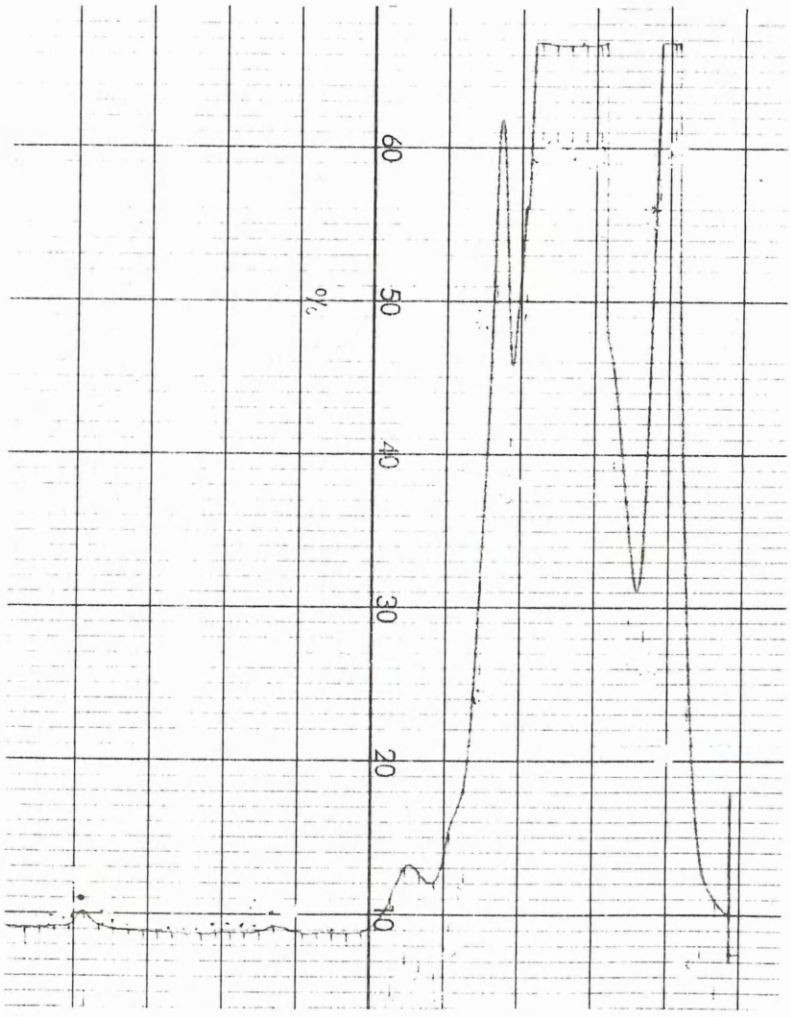
From the absorbence trace of the samples (1, 2, 3) it could be seen that in each extract of toxic strains there is a sharp peak at the point at which fraction 8, 9, 10 were obtained. This peak is completely missing in trace of medium control and of the extract of the non-toxic strains of M. ulcerans.



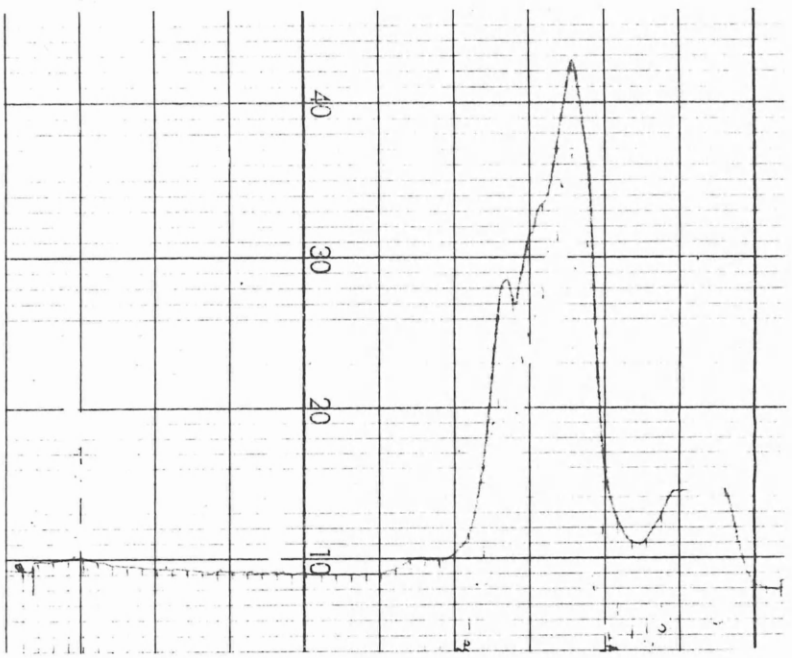
HPLC Trace of Toxic Filtrate No. 7634



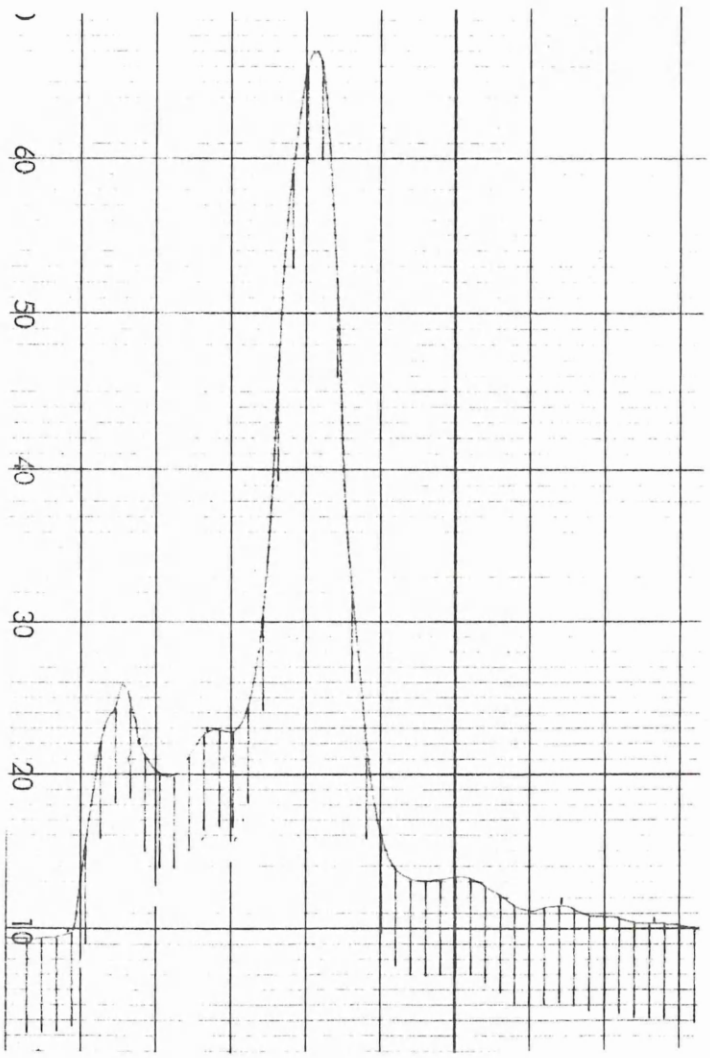
HPLC Trace of Toxic Filtrates No. 0013



HPLC Trace of Non-Toxic Filtrate No. 0009



HPLC Trace of Control Filtrates



HPLC Trace of Toxic Filtrate No. 0017

DISCUSSION

Interference with the stimulated lymphocytes by inhibition or killing of cells, is the main indicator for the presence of the toxin. The fractions numbered 8,9 and 10 obtained from filtrates of the pathogenic strain appeared to have both killing and suppressive activities similar to the results obtained before fractionation. These fractions are associated with a peak eluted at approximately 200-250kD such a peak was not present in similar preparations of non-pathogenic strains and when fractions were taken from region of the similar peak no effect on the activated lymphocytes was observed. There was no peak in the trace of the control sample showing that the toxin material did not come from the culture medium. Three other factors with lower molecular weights were also obtained by fractionation of the toxic filtrates. All of them were less than 65-kDa in size and they increased, rather than decreased stimulation of lymphocytes.

The high molecular weight of the toxic fraction show it to be a protein probably in combination with other substances.

Several investigators have combined two or more physiochemical separation techniques serially. This approach has yielded preparations in which only single constituents can be identified. The combination of gel filtration with sephadex G-200, DEAE-sephadex ion-exchange chromatography and isoelectric focusing was used to fractionate culture filtrates of M. tuberculosis by Moulton *et al* (1971). They obtained nine fractions, one of these fractions caused reactions in both sensitised and non-sensitised guinea pigs when used for skin testing.

The only description of a toxin derived from a mycobacterial species is a glycolipid containing trehalose (6,6′-dimycol-.′-D-trehalose). This substance was discovered by Bloch (1950), during petroleum-ether extracts of M. tuberculosis ($H_{37}Rv$). The toxicity of this substance was demonstrated by its ability to kill liver cells from mice in microgram amounts (Kato and Tanaka, 1967). This killing was associated with destruction of mitochondrial membrane (Kato, 1970). the ester linkages at the 6,6′ position of trehalose with mycolic acid and the presence of an hydroxyl group of the sugar in the molecule were essential for toxicity. Mycolic acid or trehalose alone or together were shown to be non-toxic for mice and non-destructive for mouse liver cells (Kato, 1968).

All of the previous work involved in separation of culture filtrates or cell wall extracts from mycobacteria is based upon findings of antigenic substances which stimulate the immune system. As a result, it was difficult at this stage to compare the results of the experiment reported in this chapter with earlier work, but my findings may make possible further studies to explain the chemical nature of the toxin.

CHAPTER EIGHT

INTRODUCTION

Although much work has been done in the past on the pathogenicity of mouse infection with M. ulcerans, few papers concentrate on the immunological response to this infection.

The cellular response to experimental Mycobacterium ulcerans infection in mice has been studied by Rook, 1975 and was described as having three different immunological stages.

First, early in the infection (2-5 weeks after inoculation), mice develop positive delayed hypersensitivity reactions together with positive leukocyte migration and lymphocyte transformation.

The second stage occurs when delayed hypersensitivity reactions and leukocyte migration responses diminish, but lymphocyte transformation persists with large numbers of responsive cells in lymph nodes.

In the third stage, the T-cells have been exposed to excessive amounts of antigen leading to the loss of lymphocyte transformation as well. This is quite different from the human disease in which mortality is a rarity. The change from non-reaction to reaction that occurs in man does not take place in the mouse when the bacteria are killed by immune mechanism during the recovery phase.

Two experiments were carried out in mice to investigate whether immunity could be induced that protected mice from developing disease after a foot-pad challenge. In the first experiment soluble antigen in Freund's incomplete adjuvant were investigate and in the second experiment the toxin and suspension of whole live bacilli were investigated.

MATERIALS AND METHODS

The preparation of antigens, the method of lymphocyte transformation tests and the preparation of single cell bacterial suspensions have been described in earlier Chapters (4,5,6). Aliquotes of 1mL PBS buffer containing the organism at the appropriate dilution were made in Eppendorf tubes and stored at -70°C.

Antigen preparation with incomplete Freund's adjuvant for Experiment Number 1:-

The adjuvant solution consists of 5mL of Arlacel A (Sigma product No. A8009) and 17mL of heavy liquid paraffin (BDH product no. 29437) mixed together. Equal amounts of previously prepared antigens (see earlier chapter 5) and adjuvant solution were mixed together using a 5mL syringe until a thick white emulsion was produced. The preparation was stored at +4°C.

Immunosorbent Assay (ELISA) Procedures:-

1. Preparation of BCG as described in Chapter 5. The antigen was diluted in coating buffer at optimum concentration of 10ug/ml and 200ul of the antigen were used to coat micro-ELISA plates (NUNC/immunoplates Maxisorp from Inter Med, catalogue no 4-42404). The plates were incubated at +4°C overnight.

2. The plates were washed three times with PBS-Tween, each time the wells were filled completely with the buffer and left for three minutes, then the buffer was discharged.

3. All sera are diluted to a concentration of 1/1000 in washing buffer, 200ul from the diluted serum was dispensed in the appropriate wall. The plates were incubated at room temperature for three hours.

4. The serum was washed off as in Step 2.

5. Rabbit immunoglobulin peroxidase conjugated to mouse immunoglobulin made by DAKO was diluted at concentration 1/1000 in washing buffer. 200ul was dispensed in each well. The plates incubated for three hours at 4°C.

6. The conjugate was washed as in Steps 2 and 4.

7. The colour reaction developed by adding 200ul of substrate to all wells and the plates incubated in the dark for half hour at room temperature (preparation of the substrate will be described later in the chapter).

8. The reaction was stopped by adding 50ul of stopping solution to each well (preparation of the stopping solution will be described later in the chapter).

9. At this stage, the antigen-antibody binding is reflected as a colour change. This was measured by reading the absorbance using a spectrophotometer at 490M wavelength, the system consisted of Micro ELISA Autoreader MR58, (a Dyntech product) and an operating function control panel.

ELISA Buffers Preparations

Substrate:-

a) 2,2-Azino-di-(3-ethyl benzthiazoline sulfonic acid)
(Sigma product No. A-1888)

b) Citrate/Phosphate buffer (0.1M) pH4.1
Solution A 0.1m Citric acid = 21.01g/l
Solution B Disodium phosphate ($Na_2HPO_4.2H_2O$) = 35.6g/l
Mix solution B with solution A at pH 4.1

c) Hydrogen peroxide (20 vol)

Substrate was made up freshly each time and used immediately.

50mg of (a) + 100mL (B) + 35ul (C)

Stopping Solution:-

Sodium fluoride (Sigma product No. S-1504) in distilled water (0.48mg Naf + 25ml H_2O)

Washing and Incubation Buffer (PBS-Tween, pH 7.4)

For one litre: distilled water

8g/NaCl

0.2g/KH_2PO_4

1.135g/Na_2HPO_4

0.2g/KCl

0.5/Tween 20

Coating Buffer (0.5m carbonate-bicarbonate pH9.6)

For one litre: H_2O

1.59g Na_2CO_3

2.93g $NaHCO_3$

EXPERIMENT NO. 1

Five groups (A to E) of 10 BALB/C mice aged 7 to 10 weeks were taken for this experiment.

Group A: These were control mice neither immunized nor challenged.

Group B: Non-immunized control mice challenged with pathogenic strain No. 7748.

Group C: Non-immunized control mice challenged with non-pathogenic strain No. 0009.

Group D: Mice immunized with adjuvant + antigen of non-pathogenic strain No. 0009 and challenged with pathogenic strain No. 7748.

Group E: Mice immunized with adjuvant + antigen of pathogenic strain No. 7748 and challenged with the same strain.

The immunizing injection was prepared from filtered sonicate of pathogenic strain No. 7748 and non-pathogenic strain No. 0009 (1.4mg protein/mL).

Equal volume of each were emulsified with Freund's incomplete adjuvant. Doses of 0.1mL were given weekly for four weeks by the intraperitoneal route to groups D and E. One week after the last immunizing injection all groups B, D and E were challenged with live pathogenic strain No. 7748.

The mice in group C were challenged with non-pathogenic strain No. 0009. The challenge was given as an injection of 0.1mL in to the left foot-pad containing 5×10^4 organism approximately. Immediately prior most of the mice were bled by cardiac puncture and one mouse in each group was killed, its spleen removed. Five weeks after challenge further blood samples were obtained from each group and a second mouse was killed and its spleen removed. At the same time the animals in

group C which had been challenged with the non-pathogenic strain were re-challenged in the right foot-pad.

Two weeks later the disease (week six from the initial start of the experiment) had progressed so far in animals of group B that the whole group were killed at this time. Four weeks after group C had been re-challenged with the pathogenic strain the disease developed and the mice were killed after further blood sample had been taken. In the remaining two challenged groups D and E the disease has progressed much more slowly and did not need to be killed until 10 weeks after. The experiment was then ended and the animals of group A were killed at this time.

In groups B, D and E the infected foot-pad was measured at weekly intervals with a spring loaded micrometer after the first appearance of swelling in group B. The micrometer was graduated in thousand of an inch. A summary of the whole experiment is described in Table 1.

TABLE NO. 1: PROTOCOL OF EXPERIMENT NO. 1

Weeks	Control Group A	Group B	Group C	Adjuvant + 0009 Group D	Adjuvant + 7748 Group E
1				First immunization	
2				Second immunization	
3				Third immunization	
4				Fourth immunization	
5	Bleed 1 - Bacterial challenge				
6	∧				
7	|				
8	|				
9	Foot-pad Readings	|Animal killed|Re-challenge| ---------- Bleed 2			
10	|				
11	|				
12	∨			Appearance of the disease	
13			Bleed 3 --------- Appearance of disease		
14	Experiment ended and all animals killed				

RESULTS OF EXPERIMENT NO. 1

The response of lymphocytes during the first stage in Experiment No. 1 (Bleed 1) demonstrate the exposure of the animals used in this experiment to the environmental mycobacteria. This can be seen from the stimulation index of Group A, B, C Table 2. Group D and E produced significant response in comparison to the other groups as a result of the immunization (Table 2). There is no statistical significance between the non-immunized groups (A, B and C) or between the immunized groups D and E, but a high statistical significance $P < 0.0001$ can be observed when the groups from the immunized mice (D and E) are compared to the groups from the non-immunized mice (A, B and C).

TABLE NO. 2: LYMPHOCYTE TRANSFORMATION DATA ON EXPERIMENT NO. 1 - BLEED 1

Groups	Lymphocyte Stimulation (Mean/SD) from First Bleed	
		Stimulation Index
A	1711 ± 891	11
B	1656 ± 997	11
C	1782 ± 1023	12
D	9986 ± 5321	62
E	14877 ± 7316	93

- total count of non-activated lymphocytes = 160
- the results are expressed as a mean/SD of CPM and stimulation index
- BCG antigen was used to stimulate lymphocytes at a concentration of 5µg/mL

Four weeks after bacterial challenge (Bleed 2) a significant increase of lymphocytes activities occurred in all groups, $\rho<0.0001$, except for control group A which had no significant increase when compared to the results before the challenge (Table 3). In Group B the pathogenic strain 7748 was used. The stimulation index increased by approximately four fold. A high statistical significance can be found between group B and C, $\rho<0.0001$, group B being higher than group C. This difference could be an individual variation between animals or a real difference between the pathogenic and non-pathogenic strains used to challenge each group. In Group C the animals challenged with non-pathogenic strain 0009, the stimulation index increased by two fold. In Group D the animals challenged with pathogenic strain 7748 were previously immunized with non-pathogenic strain 0009 and adjuvant; the stimulation index showed an increase by approximately two fold. Similar events occurred in Group E despite the difference in the contents of the immunization mixture. Both of these groups had a highly statistical significance from any other group and highly statistical significance from each other. D being higher than E.

TABLE NO. 3: LYMPHOCYTE TRANSFORMATION DATA ON EXPERIMENT NO. 1 - BLEED 2

Groups	Lymphocyte Stimulation (Mean/SD) from Second Bleed	
		Stimulation Index
A	1945 ± 765	13
B	6876 ± 1382	49
C	4392 ± 1211	31
D	23531 ± 4369	169
E	16301 ± 445	140

- total count of non-activated lymphocytes = 149
- the results are expressed as a mean/SD of CPM and stimulation index
- BCG antigen was used to stimulate lymphocytes at a concentration of 5µg/mL

TABLE NO. 5 EXAMPLE OF COUNTS OF INDIVIDUAL WALLS (CPM) FROM BLEED 2 IN EXPERIMENT NO. 1

Group A	Group B	Group C	Group D	Group E
2162	5570	3398	20359	20435
2455	6687	4450	19877	19967
1175	5788	5614	21494	19472
1150	6480	6290	20494	20102
1173	8810	4344	31705	19503
3282	6988	4519	31358	18837
2168	7752	3998	21023	19344
1159	7768	4399	21817	19297
1138	6821	3361	20696	19345
2128	8961	5812	26491	19318
3189	4000	4947	-	-
2161	-	1572	-	-

The third bleed which was taken from Group C only after they had been re-challenged with pathogenic strain showed a slight increase int he response of lymphocytes (Table 4) but this was not statistically significant. Group C in Table 3 and group C in Table 4

TABLE NO. 4: LYMPHOCYTES TRANSFORMATION DATA ON EXPERIMENT NO. 1 - BLEED 3 GROUP C

Lymphocytes Stimulation (Mean/SD) from the Third Bleed	
7113 ± 6250	Stimulation Index
	51

- total count of non-activated lymphocytes = 178
- the results are expressed as a mean/SD of CPM and stimulation index
- BCG antigen was used to stimulate lymphocytes at a concentration of 5µg/mL

The antibody responses for the first bleed (table 6) behaved in a similar manner to the lymphocytes activities. The two vaccinated Groups D and E showed a high titre of IgG but a much lower titre of IgM, approximately similar to that of the control Group A.

The levels of IgG and IgM in the Groups B and C had no significant differences than the control Group A.

TABLE NO. 6: (MEAN/SD) OPTICAL DENSITY READING OF ELISA (x 1000) FROM BLEED 1

Groups	Antibody Levels from the First Bleed	
	IgG	IgM
A	10 ± 0.8	13 ± 1.5
B	12 ± 2.2	17 ± 1.8
C	14 ± 3	18 ± 2.0
D	210 ± 4.2	16 ± 0.7
E	245 ± 3.3	10 ± 1

- BCG antigen was used to coat the plates at a concentration of 10µg/mL
- The serum dilution used was 1:1000

In Bleed 2 (Table 7) prior to the bacterial challenge no significant increase in the levels of IgG and IgM were found in all challenged groups.

The re-challenge for Group C with the pathogenic strain induced a slight increase in the levels of IgG and IgM (Table 8).

TABLE NO. 7: (MEAN/SD) OPTICAL DENSITY READING OF ELISA (x 1000) FROM BLEED 2

Groups	Antibody Levels from the Second Bleed	
	IgG	IgM
A	5 ± 0.7	8.3 ± 0.9
B	16.5 ± 1.5	22.5 ± 2
C	11.5 ± 1.3	11.5 ± 1.4
D	313 ± 6.6	10 ± 1.0
E	301 ± 5.4	7 ± 0.07

- BCG antigen was used to coat the plates at a concentration of 10µg/mL
- The serum dilution used was 1:1000

TABLE NO. 8: (MEAN/SD) OPTICAL DENSITY READING OF ELISA (x 1000) FROM BLEED 3 GROUP C

Antibody Levels from Bleed 3	
IgG	IgM
34 ± 1.7	25 ± 3.2

- BCG antigen was used to coat the plates at a concentration of 10µg/mL
- The serum dilution used was 1:1000

Taking 69 and above as indicating the onset of disease (Table 10 shows that group B animals showed signs of diseases two weeks after challenge whereas both group D and E animals began to show disease five weeks afte challenge. This has a statistical signficance <0.001. Swelling continued until the animals in group B had to be killed four weeks after challenge whereas animals in group D and E did not have to be killed until seven weeks after challenge. Moreover, the foot-pad measurement of group D remained non significantly different until week 10.

Thus both Group D and E showed in delayed onset of the disease there is a distinct difference between them, and the greatest delay found in Group D. This group had been immunized with the sonicate of non-pathogenic strain. However, in all three groups once disease had started it progressed at the same rate than in the animals that had to be killed (Table 9 and 10).

TABLE NO. 9 FOOT-PAD READING TAKEN DURING EXPERIMENT NO. 1
Between week 6 and 12 for Groups B, D and E.

The results are expressed as mean/SD of thousandths of an inch. The average size of uninfected foot-pad is between 62/1000 to 57/1000, and I have taken the volume of 69/1000 and above as identification of swollen foot-pad.

Weeks	Group B	Group D	Group E
6	65 ± 1.8	64.2 ± 2.89	63 ± 1.4
7	69 ± 1.98	64.7 ± 2.2	65 ± 2.7
8	72 ± 2.1	65.5 ± 2.5	66 ± 3
9	73.5 ± 2.5	67.2 ± 1.98	68 ± 1.1
10	-	69.2 ± 1.1	70 ± 2.4
11	-	70.2 ± 1.6	71 ± 3.1
12	-	72.2 ± 2.7	73 ± 2.8
Statistical student T test			

Group D	Group E	Group B	
Week 6 v week 7 not significant	Week 6 v week 7 not significant	Week 6 v week 7 < 0.02	
Week 6 v week 8 not significant	Week 6 v week 8 < 0.02	Week 6 v week 8 < 0.001	
Week 6 v week 9 not significant	Week 6 v week 9 < 0.001	Week 6 v week 9 < 0.001	
Week 6 v week 10 < 0.001	Week 6 v week 10 < 0.001	Week 7 Group B v Week 6 Group D not significant	
Week 6 v week 11 < 0.001	Week 6 v week 11 < 0.001	Week 7 Group B v Week 7 Group E < 0.02	
Week 6 v week 12 < 0.001	Week 6 v Week 12 < 0.001	Week 9 Group B v Week 10 Group E	
Week 10 v week 12 < 0.03	-	-	-

TABLE NO. 10: EXAMPLE FROM THE INDIVIDUAL FOOT-PAD MEASUREMENTS OBTAINED IN EXPERIMENT NO. 1 BETWEEN WEEK 6 TO 12 FOR GROUP D

Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12
62	63	63	65	70	69	72
64	66	69	70	69	71	70
66	65	66	69	68	68	69
61	68	67	68	67	70	73
60	60	65	69	69	-	75
68	67	62	66	70	72	77
63	66	67	64	71	69	70
65	63	69	67	70	73	-
69	65	62	-	-	-	-

Note: The measurement for each mouse each week are random ie. the top line of readings is not all for the same mouse.

EXPERIMENT NO. 2

The second experiment was designed to investigate the possibility of immunization against M. ulcerans using a different route of inoculation. Using live bacilli, five groups of ten BALB/C mice aged between 7-8 week received different inoculations into the scruff of the neck. The can be arranged as follows:

Group A : Non-immunized and non-challenged control group.

Group B : Immunized with a standard human dose of BCG Glaxo vaccine containing 10^6 viable units.

Group C : Immunized with 0.1mL PBS suspension of strain 0009 non-pathogenic M ulcerans approximate dose of 5×10^6 organisms/mL.

Group D : Immunized with 0.1mL suspension of strain 0009 in toxic filtrate of strain 7748 (pathogenic). The approximate dose given was 5×10^6 organisms/mL.

Group E : Immunized with PBS suspension of strain 7748 at an approximate dose of 5×10^6 organisms/mL.

From pilot experiments and from Experiment 1 I found that my challenge dose produced disease in non-immunized animals after four weeks. This control was from this experiment because of shortage in suitable animals.

Pooled sera from each group were obtained seven weeks after inoculation and spleen cells were taken from two mice in each group to measure cellular responses in lymphocyte transformation tests (LTT).

At Week 8 all animal groups except the control Group D were challenged in the right foot-pad with 0.1mL live bacilli of M. ulcerans strain 7748 at an approximate dose of 5×10^6 organisms/mL.

At Weeks 9, 8, 10 and 11 sera were collected and pooled from each group and 9 week spleen cells from two mice in each group were taken for LTT.

The remaining mice were observed for development of the disease.

A summary of the experiment is described in Table 11.

TABLE NO. 11 PROTOCOL OF EXPERIMENT NO. 2

	Control	BCG	0009 Strain	0009 + toxin	7748 Strain
Weeks	Group A	Group B	Group C	Group D	Group E
1		immunization			
7	Bleed 1 and spleen				
8		bacterial challenge			
9	Bleed 2 and spleen				
10	Bleed 3 and spleen				
11	Bleed 4				
12		observation of the disease in Groups C and E			
13		observation of the disease in Group B			
14		observation of the disease in Group D			

RESULTS OF EXPERIMENT NO. 2

The immunization with different strains of M. ulcerans (pathogenic and non-pathogenic) in the scruff of the neck produced a lower stimulation in LTT than did the challenge in the foot-pad with these organisms in Experiment No. 1. This probably reflects the differences between subcutaneous challenge (neck) and the intradermal challenge (foot-pad) which is known to exist. However, the addition of the toxin supernatant to the non-pathogenic strains in Group D significantly increased the response (Table 12). This also can be observed in the levels of antibody titre (Table 13). The higher stimulation index and antibody titre seen after BCG vaccination in Group B is probably because this is also the antigen used in LTT and ELISA.

From the control results in Experiment No. 1 and other pilot experiments with M. ulcerans, infection in the foot-pad with Strain 7748 develops within 3-4 weeks after the challenge. This information was used as a standard in the second experiment.

All four vaccinated groups delayed the outset of disease. Both Groups C and E, immunized with non-pathogenic and pathogenic respectively, developed the infection within a period of four weeks from the time of the challenge (Table 11) by at least two weeks compared with expected time of onset in non-vaccinated animals (See group B in experiment No.1).

TABLE 13 ELISA RESULTS FROM EXPERIMENT NO. 2
Sera from Bleed 1, 2, 3 and 4 - BCG antigen was used to coat the plates at a concentration of 10ug/mL. The serum dilution used was 1:1000 and results are expressed as mean/SD of OD x 1000.

	Antibody Levels			
Groups	Week 7	Week 9	Week 10	Week 11
A. Control Group	10 ± 1.6	11 ± 3	11 ± 1.8	12.5 ± 3
B. BCG Group	89 ± 6.1	88 ± 5	90.8 ± 5	98 ± 6
C. Group vaccinated with Strain No. 0009	55 ± 3.0	58 ± 10	59 ± 3.6	55.5 ± 14
D. Group vaccinated with Strain No. 0009 + toxin	97 ± 11.9	97.5 ± 6.8	100 ± 8	100 ± 5.8
E. Group vaccinated with Strain No. 7748	50.4 ± 2.9	53.3 ± 4.2	60 ± 2.86	60.1 ± 5.3

DISCUSSION

Attempts to develop a vaccine against tuberculosis with specific antigen have not yet succeeded and the whole live attenuated BCG bacilli are still in use. However, the BCG trials (described in Chapter Two) have shown that the protection reported in some parts of the world is not reproducible in other areas.

In the first experiment, I used filtered sonicated organisms coupled with adjuvant to immunize against the disease, but only a few weeks delay were observed before the appearance of the infection. Immunization with sonicated pathogenic and non-pathogenic organism appeared to produce the same effect of delay. Immunization with non-pathogenic strains in the foot-pad do not result in preventing the infection when the mouse is challenged in the other foot-pad.

The approach in preventing the disease in the second experiment used a different route of immunization (subcutaneous) and compared the abilities of four different vaccination procedures to delay the onset of disease after the challenge. All four worked to some extent but BCG andnon-pathogenic and soluble toxin were the most effective. Fenner (1957) observed that mice given BCG were well protected against low dose but not high dose challenge with M. ulcerans. Fenner does not state the size of his challenging dose but from his description my challenge dose will be similar to his high dose challenge. Human immunization with BCG in Uganda produced an over-all short lived protection (Uganda Buruli Group 1969; Smith *et al*, 1976). The delay in

onset that I have observed in my experiment might be thought of as the equivalent in mice similarly of short lived protection seen in man.

Adding the toxic supernatant to the non-pathogenic strain considerably improved the cellular and antibody responses and further postponed the disease. This suggests that immunization against the disease using Strain 0009 may be a viable option, especially if a lower challenge dose than 5×10^5 organisms were used or a different animal model system was employed. The soluble toxin used in this experiment did not cause local necrosis to mouse tissues suggesting that an effective immunizating dose may be less than the necrotizing dose.

It appears that a balanced immune response is required to develop immunity against the infection. The delay in the development of the disease seen in this experiment may be due to a protective mechanism later overcome by the large inoculum in a highly susceptible species. A possibility that I would like to be able to investigate is the addition of the soluble toxin to BCG as a vaccine combination and to titrate the amount of soluble toxin needed to produce optimal protection.

CHAPTER NINE

THE PATHOGENIC AND NON-PATHOGENIC M. ULCERANS

Most species of mycobacteria are free-living in our surroundings. As environmental organisms they are particularly associated with warm and wet conditions (Barker et al, 1972). They appear to favour acid or neutral conditions and both fewer species and smaller total numbers of organisms occur in alkaline swamplands (Stanford and Paul, 1973). Lesser numbers inhabit moist soil and leaf mould and smaller numbers of a restricted range of species are present in dry soil. Even soil shaken from the roots of desert grasses will contain one or two slow growing mycobacterial species, usually scotochromogenic (Stanford and Paul, 1973). There is almost no situation other than sun-baked sand where mycobacteria do not live. As might be expected with such ubiquitous organisms, they have experienced little difficulty in adapting to the artificial environment of Man, and perhaps especially our domestic water system in which M. chelonei, M. kansasii and M. xenopi seem to be more frequent than they are reported to be in the natural environment (McSwiggan and Collins, 1974). The importance of such species was related to their opportunist pathogenicity for Man, or animals economically important to Man, but more attention has been given to the environmental mycobacteria since the experimental evidence has demonstrated that the mycobacterial common antigens play an important role in protection from disease. BCG has been shown to protect mice from M. lepraemurium and M. avium (Lefford et al, 1980), and M. avium-intercellulare has been shown to protect guinea pigs from

M. tuberculosis infection (Edwards et al, 1982). M. vaccae and M. non-chromogenicum have been shown to protect mice from M. kansasii infection (Rook and Stanford, 1981), and an even more important role for the environmental mycobacteria has been their association with the protective role of BCG vaccinations. This has been used to explain the results of different BCG trials (British trial 1963; Uganda BCG trial 1974; Burma trial 1972: South India trial 1987; Alabama and Georgia trials 1966).

Conclusions on the role of environmental mycobacteria were made by Stanford *et al* 1973, 1978, 1980, and Paul *et al* 1975. The multiple skin testing system used in these studies indicated that individuals with pre-existing Koch-like responses due to excessive contact, or perhaps sub-clinical infection with certain environmental mycobacteria will have relatively little natural protective immunity and will not benefit from BCG vaccination.

Some of these studies included the Burulin reagent used as part of the assessment for BCG vaccination and protection, and considered to be an indicator for the distribution of M. ulcerans.

Eshetu and Stanford 1984, Stanford and Eshetu 1983 carried out a skin test survey on school children in Ethiopia to determine the sensitisation pattern in those children in relation to their exposure to the environmental mycobacteria. Burulin was among the reagents used. The strong response observed to the Burulin reagent in one of the areas they studied was difficult to explain at the time because

there was no evidence of existing or healed M. ulcerans infection in the children skin tested, and questioning failed to disclose evidence of the disease in the area. The results from this study support my theory which describes a non-pathogenic form of M. ulcerans.

Furthermore, in this context non-pathogenic M. ulcerans and the possibility of their environmental existence as an a-virulent species would provide a basis to explain the reports since the disease was first recognized. All reported cases are either individual ones or small sporadic outbreaks in specific areas. My explanation for this epidemiological distribution of the disease is that variant pathogenic strains may be present in the environment in small numbers and are responsible for the sporadic or individual human infection. Moreover, the reservoir of the organism has never been found and this may be because of the expectation based on M. ulcerans isolated from clinical cases that it should cause progressive infection in mice. This concept can easily miss the majority of strains if they are of the non-pathogenic type. This is especially so because M. ulcerans has few positive biochemical features by which it can be recognized. Another important piece of indirect evidence for a possible difference between the environmental and clinical isolates of mycobacteria are the studies carried out by J M Grange 1973, Grange et al 1974, in which he described differences in the biochemical and antibiotic resistance of M. fortuitum strains isolated from the environment and from clinical cases. Grange postulated that less pathogenic variants in the environment throw off more pathogenic deletional mutant which are less able to colonize the environment and, therefore, are only found in

small numbers. Thus it is my hypothesis that M. ulcerans exists predominantly as non-pathogenic strains in the environment and only occasionally produces pathogenic variants that can produce disease if accidentally introduced into the tissue.

I had hoped to obtain evidence on the nature of the variation, but so far have not been able to do so. One possibility was that it was associated with mycobacteriophage, but I could not demonstrate phage in the few studies I did. A parallel might exist between M. ulcerans and Corynebacterium diphtheriae in this respect. Another possibility was that toxin production results from an occurring mutation, as was suggested by Grange in explanation of the production of the non-pathogenic serotype I of M. fortuitum. Several workers have observed that M. ulcerans becomes less pathogenic for mice after several passages on culture media, but that full pathogenicity returns after passage through a mouse. One explanation could be that the organism takes time to induce disease and multiplication in mouse tissue, another would be that in artificial culture the non-toxin-producing bacterial cells outgrow the toxin producing ones, so that these become progressively in a minority. What does seem certain is that toxigenicity can be lost since all my strains originated from patients demonstrating the clinical signs of the disease.

This has fulfilled the criteria for the toxin being responsible for the signs of the disease proposed by Heyningen 1955 and quoted on page 2 of my opening chapter. Ulcerans toxin is exotoxin of large molecular size. It appears to be cysolytic in intact tissue as shown by ulceration in mice and guinea pigs, but it also interferes with cellular activity in low concentration as shown by its suppression of lymphocytes transformation to mitogen and antigen.

PROTECTION AGAINST M. ULCERANS AND OTHER MYCOBACTERIA

The term "angry macrophages" or allergic state was given by MacKaness 1970 to describe monocytes activated by exposure to stimulations such as macrophage activation factor (MAF) released by sensitized T-cells, when they are exposed to specific antigen, either *in vivo* or *in vitro* (North, 1988). Despite activation, macrophages fail to kill highly virulent tubercle bacilli especially *in vitro* (Stokes et al, 1986). On the other hand, the levels of protection achieved in mice against M. ulcerans or other species of mycobacteria obtained by other workers vary and are difficult to compare. The main reasons are the considerable variations within experimental conditions. These include the test organism, the dose and route of vaccination and challenge, the time between vaccination and challenge and the genetic susceptibility of the host species. Moreover, the relationship which can be developed between these experimental studies and infection in humans, especially with M. ulcerans, are quite uncertain.

Anergy occurs in M. ulcerans infection, the patient passes through a Burulin positive phase in the latent period before the disease becomes clinically obvious. This is followed by a Burulin negative phase of variable length during which the disease is at its most destructive. At this time there appears to be no immunological recognition of, or reaction to, the highly cytotoxic organisms in the lesions, although there are certainly antigen reactive cells present in lymph nodes (Rook 1975). The anergic phase, which may last several months, is

followed by a period of recovery in which the patient become strongly positive to Burulin. During the Burulin negative phase there is no reaction to PPD, which also becomes positive in the healing phase (Stanford et al, 1975).

In mice the anergic phase is associated with severe depletion of lymphocytes. This may be a result of suppression of T-cells by overloading with antigen in spleen and lymph nodes (Rook 1975), or it may be a specific effect of the toxin. How this is overcome in man with the development of the allergic healing phase of the disease is unkown but it may be due to the nutralizing effect of unidentified antibodies. The period of recovery seen in human infection does not occur in mice. This is probably because the required interaction of infected phagocytes with immunocompetent T-cells in the draining lymph node and spleen is not occurring (Collins and Watson, 1979) but some conclusions can be drawn from experimental infections of the mouse described in the previous Chapter. The early stages of the infection when the mice develop positive leukocyte migration and lymphocytes transformation (Rook 1975) can be extended for several weeks using the vaccines prepared and described in the previous Chapter. Experimental infection with small intravenous doses of M. kansasii (10^6) showed a delay in the proliferative response which coincided with a later peak in both anti-listerial and anti-tuberculosis resistance (Hepper and Collins, 1984). Adaptive transfer studies involving the use of T-cell depleted recipients infected with M. kansasii indicated that immune T-cells did not appear within the spleen of these mice until at least 60 days into primary infection (Hepper and Collins, 1984). The concept of

late T-cell responses may be applicable to the recovery stage seen in human infection, and the early proliferative response caused by the vaccine described in mice in the last Chapter. This unfortunately does not lastingly prevent infection, although the situation could easily be different in other animal species. Delaying of the onset of the disease by vaccination is sometimes seen in animal models where the challenge dose is large. A much smaller and more realistic challenge dose imposes many problems in experimental infection in animals, but would have provided a much better test of the true vaccinating effect of my preparations.

My experiments in mice have provided some preliminary evidence that an improved vaccine against M. ulcerans infection might be based on a combination of the toxin with a non-toxic organism. Although I used a live non-pathogenic M. ulcerans strain, to which I added the toxin, the next experiment might be to replace the non-pathogenic strains with BCG, and perhaps to convert the toxin into a toxoid which might have the same immunological effect, but lack the tissue damaging effect of the actual toxin. There is no shortage of further experiments that might be carried out in this field. If a simple addition to BCG could be developed that could be used in regions where M. ulcerans disease is a problem, the cost of its employment might not be too great, in comparison with the cost of treating the disease.

Toxins of M. ulcerans

The knowledge of the precise role of toxins in diseases is not always a condition of their use as protective antigen, nor is it usually necessary to purify and characterise them before use. Sometimes empirical vaccines consist of mixtures of relatively crude fractions made up as toxoid. These can be extremely effective protective agents such as the clostridial toxoid used in veterinary medicine. In other instances little protection is provided by vaccination using this approach or by vaccination with relatively well characterised toxoids such as staphlococcal toxoid. In such circumstances, evaluation of each individual virulence factor is the overall process of pathogenicity is required.

It is not yet clear whether a single or a group of virulence factors are involved in either protection from or disease susceptibility to mycobacteria. Although BCG vaccination is the best method available to prevent tuberculosis, it is unable to alter substantially the disease, and shows variable efficacy. Today many people think there is a better return on finding and treating cases than vaccination.

From the experiments described in Chapter Four, it is apparent that the toxin of M. ulcerans plays an important role in the pathogenicity of the organism. The *in vitro* results (LTT) clearly distinguish suppressive activities on lymphocyte stimulation, and skin testing with this substance causes killing to the epidermis in non-sensitized mice and guinea pigs. Therefore the toxins effect *in vivo* could be the

suppression of protective immune mechanisms before any level of protection has been achieved in non-vaccinated mice. The toxin could also be responsible for the relatively low level of protection measured by delay of disease development in mice vaccinated with different vaccines used in Experiments 1 and 2 (Chapter Eight). In human infection the toxin's effect clearly occurs during the Burulin negative phase. At this stage the toxin and the amount of destroyed tissue around the ulcer delays the stimulation of specifically activated T-cells which are required to enter the primary lesion in large numbers, and immunologically activate the blood monocytes. The end of the toxin's activity is marked by the return of the reactivity to Burulin and the start of the recovery phase. Therefore it can be concluded that protection from M. ulcerans infections in humans is by prevention of the negative Burulin and PDD reactivity phase.

The methods developed in my research to produce the toxin have shown that it is an actively secreted substance, but have posed a major obstacle to further studies. This is mainly the limited volume of toxin produced in each batch, which means that new batches have to be prepared for each new experiment. Nevertheless, it did provide conclusive evidence of the toxic activities and using the same filtrates, the HPLC system was able to locate these activities to a particular fraction. However, it is important to obtain a higher standard of purification of the toxin than that achieved by HPLC to understand the immunological process of M. ulcerans infection.

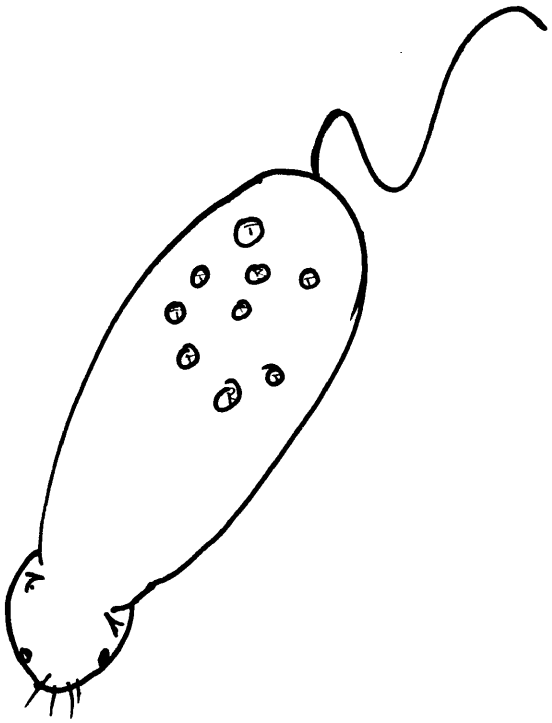
The new technology of gene cloning may provide a powerful new tool for the study of M. ulcerans and for the production of new and improved biological products for disease control. It is interesting to find that, so far, M. ulcerans has not been a subject of study by molecular biologists, except for the work reported in this thesis. Simple digestion of M. ulcerans DNA with several endonucleases and hybridisation with different probes may allow separation of this species on the basis of pathogenicity as I have suggested, or on the basis of their origin.

At the end of my study, although more has been learned about the production and activities of the toxin, and its large molecular weight has been confirmed, its chemical nature other than that it must be a protein, is still unknown.

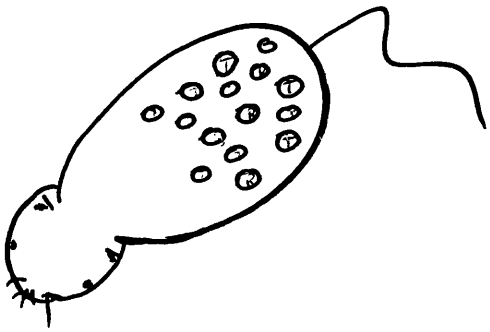
I would like to conclude with a theoretical model of what may happen in the experimental mouse.

A) Unimmunized Mouse

Large dose of M. ulcerans includes preformed toxin which kills local tissue, protecting the organism. More toxin diffuses into the circulation preventing T cell from multiplying up so no effective cellular immunity or T cell dependent antibody formation can occur. As more and more toxin is produced the lymphocytes die and the situation becomes immunologically irretrievable.



B) Optimally vaccinated mouse



Pre-formed toxin in the challenge dose may be neutralised probably by antibodies, and some bacilli may be killed, but the size of the challenge dose may be too great. Newly formed toxin may be made by surviving organisms and this may exceed the serum neutralising power and prevent further T cell and B cell replication. Once this has occurred the steady production of toxin overcome resistence and the disease ensues. This vaccin might have been effective against small challenge and the disease is simply delayed in onset.

If my suggested model is correct the measurement of delay in onset of the disease may be a true measure of vaccination power. Thus, using this simple model an optimal vaccine could be developed.

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